VANESSA ALEXANDRA MARTINS GOUVEIA

BIOREMEDIATION OF OIL ALONG THE NW PORTUGUESE COAST – THE ROLE OF AUTOCHTHONOUS MICROORGANISMS

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Orientador – Ana Paula Mucha
Categoria – Investigadora
Afiliação – Investigadora do Centro
Interdisciplinar de Investigacao
Marinha e Ambiental (CIIMAR)

Co-orientador - C. Marisa R. Almeida
Categoria – Investigadora
Afiliação – Investigadora do Centro
Interdisciplinar de Investigacao
Marinha e Ambiental (CIIMAR).

"Hoje, neste tempo que é seu, o futuro está sendo plantado. As escolhas que você procura, os amigos que você cultiva, as leituras que você faz, os valores que você abraça, os amores que você ama, tudo será determinante para a colheita futura"

Padre Fábio de Melo

"O futuro tem muitos nomes.

Para os fracos é o inalcançável.

Para os temerosos, o desconhecido.

Para os valentes é a oportunidade."

Victor Hugo

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Resumo

O derrame de petróleo causado por acidentes com petroleiros constitui uma das principais causas responsáveis pela contaminação ambiental. Por este motivo, várias técnicas de restauração foram desenvolvidas, entre elas a biorremediação. A biorremediação é agora usada extensivamente para biodegradação da contaminação por hidrocarbonetos resultante das atividades relacionadas com a indústria petroquímica, que pode remeter para completar a mineralização de contaminantes orgânicos em dióxido de carbono, água, compostos inorgânicos ou transformação de contaminantes orgânicos complexos em outros compostos orgânicos simples, através de agentes biológicos como microrganismos. Muitos microrganismos autóctones do solo e da água são capazes de degradar hidrocarbonetos. Assim, a biorremediação pode ser uma ferramenta valiosa para recuperar os ecossistemas afetados por derrames de petróleo.

O objetivo deste estudo foi compreender o potencial das comunidades microbianas para a degradação de hidrocarbonetos ao longo da Costa NO Portuguesa. Para isso, dois trabalhos diferentes foram realizados. O primeiro trabalho teve como objetivo caracterizar o potencial dos microrganismos autóctones, colhidos ao longo da Costa NO Portuguesa, para a degradação de hidrocarbonetos. Os resultados mostraram que os microrganismos degradadores de hidrocarbonetos foram encontrados em todos os sedimentos coletados, apesar dos diferentes graus de contaminação por hidrocarbonetos. Assim, nos locais selecionados, as comunidades microbianas autóctones caracterizadas têm potencial para degradar hidrocarbonetos, sendo importante avaliar experimentalmente a sua capacidade de biorremediação destes poluentes. O segundo trabalho tem como objetivo estudar o potencial dos microrganismos autóctones para a biorremediação de sedimentos contaminados com diferentes tipos de óleo. Estudos de microcosmo em laboratório foram feitos com sedimentos enriquecidos com petróleo, gasóleo ou óleo de turbina. Os resultados indicam que as comunidades microbianas autóctones têm potencial para responder à presença de diferentes hidrocarbonetos, através da alteração da estrutura dessas comunidades e aumentando o número de degradadores de hidrocarbonetos, levando à degradação (14% – 98%) dos diferentes tipos de óleo.

Portanto, biorremediação pode ser uma alternativa viável para a remoção da poluição por hidrocarbonetos ao longo da Costa NO Português.

Palavras-Chave: Microrganismos autóctones, Biorremediação, Contaminação dos sedimentos, Ambientes estuarinos e costeiro.

Abstract

The oil spill caused by tanker accidents are one of the main causes responsible for environmental contamination. For this reason, several techniques have been developed for restoration, including bioremediation. Bioremediation is now used extensively for the biodegradation of hydrocarbon contamination resulting from the activities related to the petrochemical industry, which may refer to complete mineralization of organic contaminants into carbon dioxide, water, inorganic compounds or transformation of complex organic contaminants to other simpler organic compounds, by biological agents like microorganisms. Many autochthonous microorganisms in water and soil are capable of degrading hydrocarbon compounds. Thus, bioremediation can be a valuable tool to recover ecosystems affected by oil spills.

The aim of this study was to understand the potential of microbial communities for hydrocarbon degradation along the NW Portuguese Coast. For that, two different works were performed. The first work aimed to characterize the hydrocarbon degradation potential of autochthonous microorganisms collected along the NW Portuguese Coast. Results showed that hydrocarbon degrading microorganisms were found in all collected sediments, despite the different degrees of hydrocarbons contamination. Thus, at the selected sites, the characterized autochthonous microbial communities had the potential to degrade hydrocarbons, being important to assess experimentally their ability to bioremediation of these pollutants. The second work aimed to study the potential of autochthonous microorganisms for bioremediation of sediments contaminated with different types of oil. Laboratory microcosm's experiments were carried out with sediments spiked with crude oil, diesel oil or turbine oil. Results indicated that the autochthonous microbial communities have the potential to respond to the presence of the different hydrocarbons, by changing community structure and increasing the number of hydrocarbon degraders, leading to the degradation (14% – 98%) of the different types of oil.

Therefore, bioremediation can be a feasible alternative for the cleaning of oil pollution along the NW Portuguese Coast.

Keywords: Autochthonous microorganisms, Bioremediation, Sediment contamination, Estuarine and coastal environment.

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Abbreviations

ALF	ARISA fragments lengths
ANOSIM	Analysis of similarities
ANOVA	Analysis of variance
ARISA	Automated rRNA intergenic spacer analysis
BA	Bioaugmentation
BH	Bushnell Haas
BS	Biostimulation
BSA	Bovine serum albumin
C	Carbon
CFU	Colony-forming unit
DAPI	4',6'-diamidino-2-phenylindole
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide triphosphates
FC	Fecal coliforms
FE	Fecal enterococci
FITR	Fourier transform infrared spectrophotometry
GC-FID	Gas chromatography-flame ionization detection
HD	Hydrocarbon-degrading microorganisms
HNS	Hazardous and noxious substances
IGS	Intergenic spacer
INT	Iodonitrotetrazolium violet
LOD	Limits of detection
M	Molar
MAV	Maximum admissible value
MDS	Multidimensional scaling
$MgCl_2$	Magnesium chloride
mg	Milligram
min	Minutes

Ml	Milliliter
MPN	Most probable number
N	Nitrogen
NA	Natural attenuation
NaCl	Sodium chloride
NW	Northern West
OM	Organic matter
OTUs	Operational taxonomic units
P	Phosphorus
PAHs	Polycyclic aromatic hydrocarbons
PCA	Principal components analysis
PCR	Polymerase Chain Reaction
PHC	Petroleum hydrocarbons
RMV	Recommended maximum value
rRNA	Ribosomal ribonucleic acid
Rpm	Rotations Per Minute
SPME-GC-MS	Solid-phase microextraction
SDS	Sodium dodecyl sulfate
Sec	Seconds
T_0	Time zero days
T15	Time fifteen days
TCC	Total cell counts
TAE	Tris-acetate-EDTA
TE	Tris-Edta
TPH	Total petroleum hydrocarbons
Ul	Microlitres
UV	Ultraviolet
V	Volt
ºC	Degrees Celsius
%	Percentage

Chapter 1

GENERAL INTRODUCTION

1. General introduction

Pollution by oil and its derivatives is considered a worldwide problem, becoming a more and more relevant environmental concern. The levels of petrochemical products have increased in the environment, particularly in the coastal and estuarine ecosystems. These ecosystems are ecologically very important, with a wide diversity of species, providing countless services to humans (Mucha et al. 2011; Kumar 2013). For example, oil slick forms an anaerobic condition in the sea water and leads to the death of flora and fauna. Oil spills can cause hypothermia in marine birds and mammals by reducing/destroying the insulating ability of the plumage of birds and the fur of mammals. Moreover, the toxic constituents in petroleum can poison or kill birds, mammals, fishes and other marine organisms and damage the fragile underwater ecosystems which will lead to a vicious effect on the global food chain, and eventually may harm the human health by damaging internal organs, such as kidneys, lungs, and liver (Xue et al. 2015). Leaks and accidental spills occur regularly during the exploration, production, refining, transport, and storage of petroleum and petroleum products, which have attracted great attention due to its catastrophic impact on the coastal and estuarine environment (Kumar 2013). Furthermore, these areas are subjected to large anthropogenic pressure by receiving the petroleum products resulting from domestic and industrial wastewater discharges, recreation boats by urban runoff or spill and oil leakage from boats and ships and by via pipelines losses (Ribeiro et al. 2013; Mucha et al. 2011).

In recent years, there have been several oil spill disasters: Jakob Maersk where 88000 tons of oil were spilled, Leixões, Portugal (1975); Aragon with 25000 tons of crude oil discharged in the seawater, Madeira, Portugal (1989); Exxon Valdez marked 37000 tons of crude oil spilled, Alaska, United State (1989); Sea Empress registered 130000 tons of crude oil in the sea, Wales, UK (in 1996); Erika with 31000 tons of heavy fuel oil poured, West of France (in 1999) and Prestige with 77000 tons of heavy fuel oil shed in the sea, Spain/France (in 2002) (from http://www.itopf.com/in-action/case-studies/). In addition, Coral Bulke registered 540 tons of fuel and diesel oil spilled, in the Northwest coast of Portugal (in 2000). More recently, BP Deepwater Horizon reported 548 tons oil spill,in Gulf of Mexico (in 2010) and also Rayong oil appointed with36 tons of

crude oil, in Gulf of Thailand (in 2013). This year, Refugio Oil Spill registed 80000 tons of crude oil poured in the sea, at Santa Barbara, California (2015).

Atlantic coast of the Iberian Peninsula is one of the main routes of oil cargo (Fig.1A). In the last 40 years, six major oil spills (Fig.1B) occurred in NW Iberian Peninsula as a result of tanker accidents (Ribeiro, H (2013)).

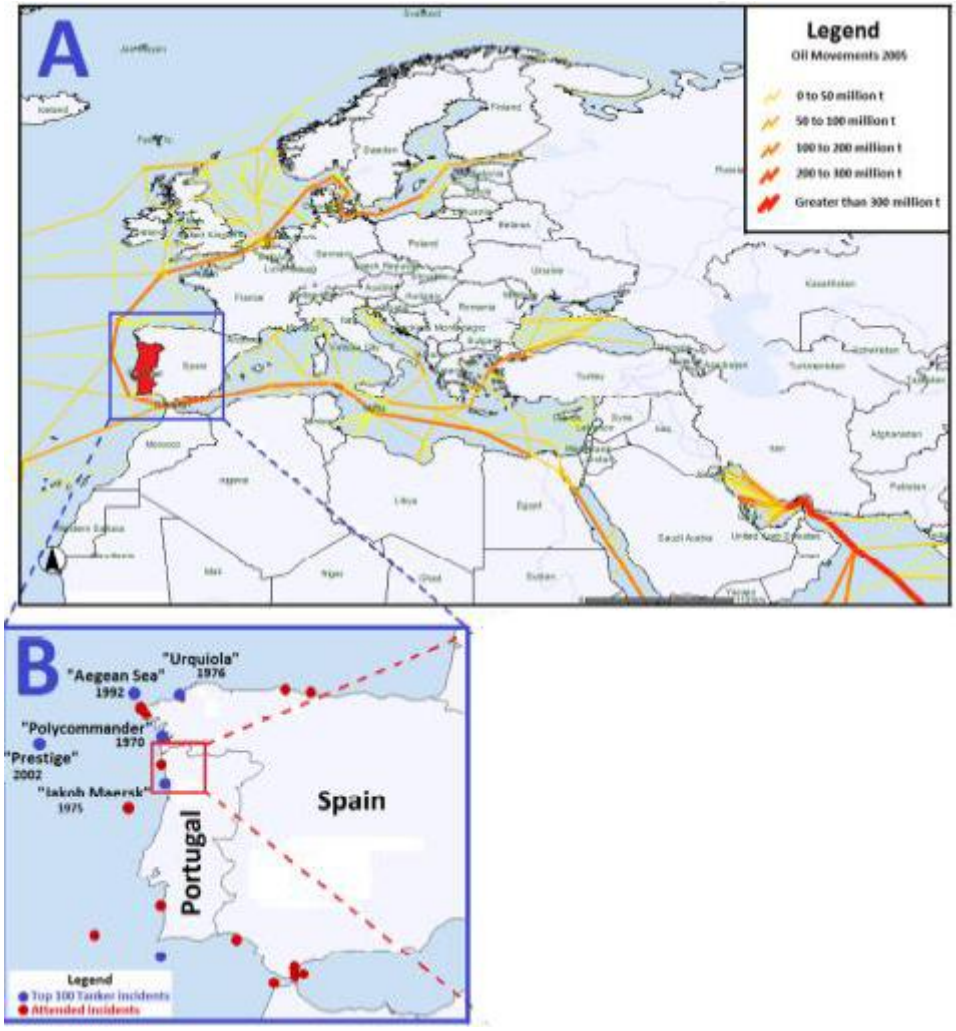


Fig. 1–A – Oil movements and amount transported in Europe during the year 2005; B – Major oil spills occurred in the coast of the Iberian Peninsula as a result of tanker accidents and

The severity of the impacts of oil spills depends on several factors such as the type of oil, the spill size, the affected ecosystem and time and meteorological conditions at the time of the spill oil (wind and waves). The sediment type also plays an important role (Kumar 2013). When an oil spillage reaches the coast, it may occur two different types of pollution: surface and subsurface. For the

subsurface pollution, two different mechanisms are described: penetration and burial (Bernabeu et al. 2009).

- The first mechanism consists in the downward percolation of oil to a depth of about 40 cm, depending on sand or sediment texture and oil type (Bernabeu et al. 2009). This mechanism is typical on beaches composed of gravel or coarse sand and it was the main mechanism of subsurface pollution in the Exxon Valdez spill (Irvine et al. 2006). Until recently, it was assumed that it was also the main mechanism of subsurface pollution of coarse beaches in other spills (Owens et al. 2008).

- The second mechanism of subsurface pollution is oil burial, described by Bernabeu et al. (2006), that showed that the morphodynamic behavior of sandy beaches has a key influence in the early stages of their oiling, determining both the initial depth of oil burial (which may reach several meters) and the extent of oiling. Bernabeu et al. (2006) studied oil contamination in the intertidal area of two beaches of the Costa da Morte (in the Galician coast) affected by the Prestige oil spill. When the accident occurred (in winter season), some beaches were extremely eroded and the oil was deposited in long layers, which were accumulated in the intertidal area. Traditional cleaning techniques were used for removing a large part of that oil. The oil that was not eliminated was subjected to the action of the wave breaking which occurs in this narrow coastal strip. The breaking phenomenon generates turbulent movements capable of stirring the sediment at the bottom. This mechanism was responsible for the initial fragmentation of the large layers of oil presented on the beaches and the active mixing between the oil and the sediment. This way, tar-balls with sediment of several centimeters in diameter were generated, which spread in the intertidal area of the beaches. In the calm conditions after the storm, the wave conditions are less energetic, favouring the transport of sediment from the lower part (subtidal) towards the higher part (intertidal) of the beach profile. The tar-balls embedded in the sediment are subjected to the pressure exercised by the sedimentary column, intensifying the oil-sediment mixture. From this point, the evolution and degradation of the oil will depend directly on the morphodynamic variability of the beach. The dynamics of the beach favours the burial-exhumation cycle of the oil and

its transport, together with the sediment, from the subtidal area to the intertidal area and vice versa. During this transport, the interaction between the oil and the sediment intensifies abrasion, where the tar-balls diminish in size progressively, until they form particles of about mean grain size.

The weathering processes, a process known that modifies the properties and composition of oil, are interlinked and contribute to oil dispersion and degradation. The main oil weathering processes include evaporation, photo-oxidation, dissolution, emulsification, adsorption, sedimentation and microbial degradation (Gong et al. 2014). All these processes, integrated into a specific environment, determine the fate and impact of the oil. Normally, after oil spill, spreading is affected by the action of winds, waves, water currents, oil type and temperature, and enhances evaporation of the volatile compounds such as low molecular weight alkanes and monoaromatic hydrocarbons, that contributes to a reduction of aliphatic hydrocarbon concentration. The lighter hydrocarbons depletion results in reduced volume oil, increasing its viscosity and density. Spilt oil is broken into droplets and dispersed through the water column, enhancing the biodegradation of hydrocarbons and dissolution of water-soluble fractions of oil. Turbulent seas cause water droplets to be suspended in the oil, resulting in water-in-oil emulsions which are difficult to degrade because of their high viscosity and reduced surface area. Photo-oxidation is the process by which hydrocarbons react with oxygen in the presence of sunlight, resulting in structural changes that, on the one hand, can lead to increased water solubility or, conversely, increased recalcitrance to biodegradation. Sedimentation will generally only occur when oil adsorbs to particles owing that to nearly all crude oils having a lower density than seawater (McGenity et al. 2012).

Therefore, it is important to develop oil clean-up strategies. At present, different cleanup and recovery methods for the oil spills have been developed including physical (e.g., controlled burning; absorbing), chemical (e.g., dispersing), and bioremediation treatments. Generally, physical and chemical treatments should be taken as emergency measures. For example, at the start of the oil spill accident, the physical and chemical treatments are used in order to rapidly control the diffusion and drift of oil. These methods are not suitable for

ecological restoration. Bioremediation is considered as one of the most important cost-effective technologies for marine ecological restoration, which leads to a complete decomposition of complex petroleum hydrocarbons of spilled oil ultimately into nontoxic compound. In the bioremediation process, appropriate microorganisms are essential for the biotransformation/biodegradation of the pollutant (Xue et al. 2015).

1.1 Bioremediation processes

Due to complexity of the petroleum and its derivatives, the treatment of contaminated areas is becoming quite difficult and problematic (Kumar 2013). Bioremediation is considered an efficient and economical cleaning option for areas affected by contaminants. Bioremediation involves the use of biological agents, particularly microorganisms that catabolize specific molecules, destroy dangerous contaminants or transform them into less harmful forms (Fuentes et al. 2014).

Bioremediation can be used as an in situ or ex situ treatment technique. Ex situ treatments involve land farming, composting and biopiles, but intrinsically linked with these technologies are excavation or removal of contaminated soil/sediments, moving the contamination elsewhere. In situ treatments consist in natural attenuation, bioaugmentation and biostimulation (Simarro et al. 2013).

Natural attenuation occurs without human intervention relying on natural conditions and behaviour of indigenous soil/sediment microorganisms (Megharaj et al. 2011). Another approach is biostimulation which improves the degradation potential of the microbial communities already present in the affected environment. This is done by adding nutrients (not available in suitable concentrations in the environment) into the contaminated site to increase the growth and metabolic activities of the indigenous microbes for the degradation of pollutants (Singh and Chandra 2014) . In fact, it has been shown that the degradation of petroleum hydrocarbons by a given autochthones microbial population can be favoured by the presence of the required nutrients in an appropriate ratio, normally C:N:P, following the Redfield Ratio. Bioaugmentation

consists in the addition of specific microorganisms, using commonly the following options (Singh and Chandra 2014):

- Addition of non-native strains or cultures. For example, inoculated strains of bacteria (*Pseudomonas*) added to contaminated soils have proven to be an efficient bioaugmentation treatment in removing contaminants without accumulation of toxic intermediates (Wang et al. 2014). From a diesel-contaminated soil, Franzetti et al. (2009) isolated the *Gordonia sp. strain BS29*. The results showed that the *Gordonia sp. strain BS29* are able to effectively remove crude oil and PAHs from soil.

- Introduction of genetically engineered microorganisms into the contaminated site via dissemination of the degradative gene from the exogenous microorganisms into the native ones also has showed positive results (Inoue et al. 2012); Singh and Subhash 2014).

- Stimulation "ex situ" of natural microbial populations which are then reintroduced into the contaminated site. The samples of autochthonous degraders are taken from the contaminated site and are stimulated to grow in the laboratory, in contact with high concentrations of the contaminant (Couto et al. 2010). Then, when a large biomass of actively growing cultures is obtained, they are inoculated at the contaminated site. Reintroduction of indigenous microorganisms can be more effective if it is supplemented with oxygen or nutrients (Couto et al. 2010).

The addition of exogenous microorganisms whether genetically modified or not into the contaminated site leads to adaption problems and environment imbalance, preventing the bacterial population to grow beyond a certain level (Joutey, Bahafid, and Sayel 2013) or arising ethic problems.

On the other hand, the use of indigenous microorganisms may be a valuable bioremediation strategy for cleaning the environment from hydrocarbon pollutants.

Therefore, the main advantage of the in situ bioremediation processes is that they allow soils/sediments to be treated without being excavated and transported, resulting in less disturbance of site activities at a relative low cost (Fuentes et al. 2014; Megharaj et al. 2011).

1.2 Petroleum and its derivatives

Petroleum is characterized by a complex combination of hydrocarbons that represent one of the most common groups of persistent organic pollutants in coastal and estuarine systems (Oliveira et al. 2014). They are of great concern because of its wide distribution, volatility, bioavailability, degradability, persistence, complex composition, and toxicity (Fuentes et al. 2014). Hydrocarbons are the principal components of a range of commercial products that derived from petroleum (e.g., gasoline, fuel oils, lubricating oils, solvents) (Oliveira et al. 2014).

The hydrocarbons are constituted mainly of hydrogen (10–14%) and carbon (83–87%). Petroleum components are classified into four groups based on their solubility differences:

- Aliphatic hydrocarbons include alkanes, alkenes, alkynes and cycloalkanes. These are the most abundant constituents of oil and more readily biodegradable. Alkanes are open chain hydrocarbons, saturated which present only simple covalent bonds (C_nH_{2n} + 2). Alkenes are unsaturated hydrocarbons, i.e., exhibit a covalent double bond between carbon atoms (C_n H_{2n}). Alkynes are hydrocarbons which have a triple covalent bond between carbon atoms without the sequence forms a cycle (C_nH_{2n-2}). The cycloalkanes have one or more saturated rings (C_nH_{2n}) (Dupuis et al. 2015).

- Aromatic hydrocarbons include monocyclic aromatic hydrocarbons, such as benzene, toluene, ethylbenzene and xylene collectively known as BTEX. They are constituents that have higher solubility in water and whose molecular structure is characterized by having a benzene ring. These hydrocarbons also include polycyclic aromatic hydrocarbons (PAHs) that have two or more condensed benzene rings that demonstrate high chemical stability due to double bonds. These pollutants are environmentally persistent and have carcinogenic properties. Some PAHs can be of difficult biodegradation. Normally these compounds are hydrophobic, hence they tend to adsorb to the soil and sediment (Hawumba et al. 2010).

- Resins (pyridines, quinolines, carbazoles, sulfoxides, and amides) and asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins) consist of non-hydrocarbon polar compounds, with trace amounts of nitrogen, sulfur and/or oxygen in addition to carbon and hydrogen (Dupuis et al. 2015).

1.3 Hydrocarbon-degrading microorganisms

Isolating and identifying microorganisms responsible for hydrocarbon transformations has long been recognized as crucial for obtaining the most promising strains for site decontamination (Bučková et al. 2013).

Since the middle of the last century, the use of petroleum degradation microorganisms for spilled oil bioremediation has been widely reported. Several bacterial genera are known to degrade or transform hydrocarbons and use hydrocarbons as a sole source of carbon and energy. The phylogenetic diversity of hydrocarbon-degrading microorganisms (HD) is vast, and several recurrent groups are found in both marine and soil/sediment environment (Xue et al. 2015). Examples of hydrocarbon-degrading bacteria are presented in Table 1 (Chikere et al. 2011).

Table 1–Genera of bacteria that utilizes hydrocarbons as sole source of carbon and energy (adapted from Chikere et al. 2011).

Genus	Typical substrate	Genus	Typical substrate
Achromobacter	Gas oil	Klebsiella	Crude oil
Acidecella	Naphthalene	Lactobacillus	Crude oil
Acidovorax	Phenanthrene	Leclerica	Pyrene
Acinetobacter	Gas oil	Leucothrix	Crude oil
Actinomyces	Crude oil	Lutibacterium	Phenanthrene
Aeromonas	Diesel oil	Marinobacter	Crude oil
Agrobacterium	Gasoline	Micrococcus	Hexadecane
Alcaligenes	Gas oil	Moraxella	Biphenyl
Alcanivorax	Alkanes; crude oil	Mycobacterium	Phenanthrene
Alkanindiges	Alkanes	Neptumonas	Naphthalenes
Alteromonas	Crude oil	Nocardia	Alkanes; crude oil
Arthrobacter	Gas oil	Nocardioides	Phenanthrene; crude oil
Aureobacterium	Crude oil	Ochrabactrum	Diesel
Azoarcus	Toluene	Oleiphilus	Alkanes
Azospirillum	Jet fuel	Oleispira	Alkanes; crude oil
Azotobacter	Crude oil	Paenibacillus	Phenanthrene
Bacillus	Toluene; crude oil	Pasteurella	Fluoranthene
Beijerinckia	Phenanthrene	Peptococcus	Crude oil
Blastochloris	Toluene	Planococcus	Alkanes; crude oil
Brevibacterium	Alkanes	Polaromonas	Naphthalene
Brevundimonas	Fuel oil	Proteus	Crude oil
Burkholderia	Toluene	Pseudomonas	Gas oil; crude oil
Clavibacter	Naphthalene	Ralstonia	Toluene
Comamonas	Phenanthrene	Rhodococcus	Phenanthrene; crude oil
Corynebacterium	Fuel oil; crude oil	Sarcina	Crude oil
Cyclostaticus	Biphenyl; crude oil	Serratia	Crude oil
Cytophaga	Crude oil	Sphaerotilus	Crude oil
Dechloromonas	Benzene	Sphingomonas	Toluene
Desulfatibacillum	Alkanes	Spirillum	Crude oil
Desulfobacterium	Xylene	Staphylococcus	Diesel
Desulfobacula	Toluene	Stenotrophomonas	Pyrene
Desulfosarcina	Xylene	Streptomyces	Alkanes
Desulfosporosinus	Gasoline	Thalossolituus	Alkanes; crude oil
Dietzia	Alkanes	Thauera	Toluene
Enterobacter	Alkanes	Thermoleophilum	Alkanes
Erwinia	Alkanes	Thermus	Pyrene
Flavobacterium	Diesel oil; crude oil; phenanthrene	Terrabacter	Fluorene
Geobacillus	Crude oil	Vibrio	Phenanthrene
Geobacter	Toluene	Xanthobacter	Dibenzothiophene
Gordonia	Alkanes; crude oil	Xanthomonas	Phenanthrene

1.4 Mechanism of petroleum hydrocarbon degradation

The hydrocarbon degrading (HD) bacteria has been a focus of attention due to its potential for biodegradation of spilled oil, contributing for its removal, as these bacteria can use hydrocarbon as a carbon and energy source. Bacterial communities degrade these hydrocarbons by several metabolic pathways (Chikere et al. 2011). The metabolic pathways that HD uses can be either aerobic (i.e. they utilize oxygen as the primary electron acceptor) or anaerobic (i.e. they utilize an alternative electron acceptor such as nitrate or sulfate). Aerobic degradation usually proceeds more rapidly and is considered to be more effective than anaerobic degradation (Fritsche and Hofrichter 2001). One reason is that aerobic reactions require less free energy for initiation and yield more energy per reaction. Thus, present work will be focus on aerobic degradation.

Generally, petroleum compounds are biodegraded, following three processes (Fig.2). At first, petroleum compounds are adsorbed onto microbial surface; second, these petroleum compounds are transferred through the microbial cell membrane; then, these compounds are degraded inside the microbial cell. At last, these compounds are degraded into various small molecules by microorganisms (Fuentes et al. 2014). The degradation pathways of different petroleum compounds are different, due to their different structures (Xue et al. 2015).

The most rapid and complete degradation of the majority of organic pollutants is brought under aerobic conditions. Fig.2 shows the main principle of aerobic degradation of hydrocarbons. The initial intracellular attack of organic pollutants is an oxidative process and the activation, as well as incorporation of oxygen, is the enzymatic key reaction catalyzed by oxygenases and peroxidases. Peripheral degradation pathways convert organic pollutants step by step into intermediates of the central intermediary metabolism, for example, the tricarboxylic acid cycle. Biosynthesis of cell biomass occurs from the central precursor metabolites, for example, Acetyl coenzyme A (acetyl-CoA), succinate, pyruvate. Sugars required for various biosyntheses and growth are synthesized by gluconeogenesis (Das and Chandran 2011).

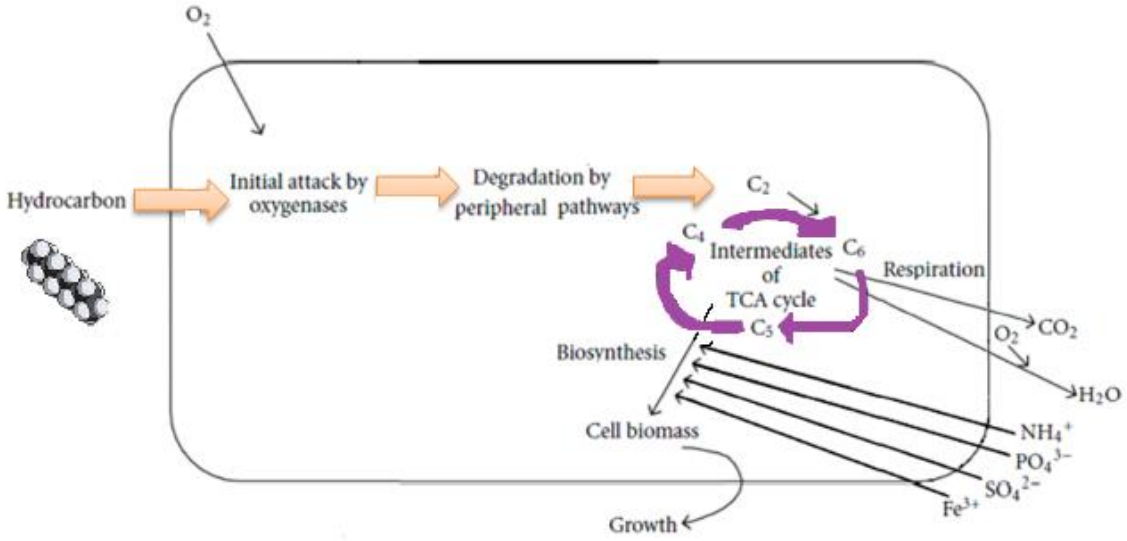


Fig. 2–Main principle for aerobic degradation of hydrocarbons by microorganisms (adapted from Das and Chandran 2011)

❖ Degrading Process of Alkanes and Cycloalkanes

The degrading process of alkanes by microorganisms is shown in Fig.3. Basically, alkanes are catalyzed by some enzymes (i.e., monooxygenases or dioxygenases) leading to the formation of alcohol. The alcohol is further oxidized to the corresponding aldehyde, in which is subsequently converted by oxidation into fatty acids, and then gradually metabolized to acetyl-CoA. Subsequently, these compounds are metabolized via the tricarboxylic acid cycle (TCA cycle) to CO_2 and H_2O (Xue et al. 2015). During degradation, cycloalkane is oxidized to alcohols by cyclohexane monooxygenase. Then, the alcohols are converted to ketone by cyclohexene dehydrogenase. Later, the ketone is oxidized to esterase or/and fatty acid. At last, the compound biodegraded into CO_2 and H_2O (Xue et al. 2015).

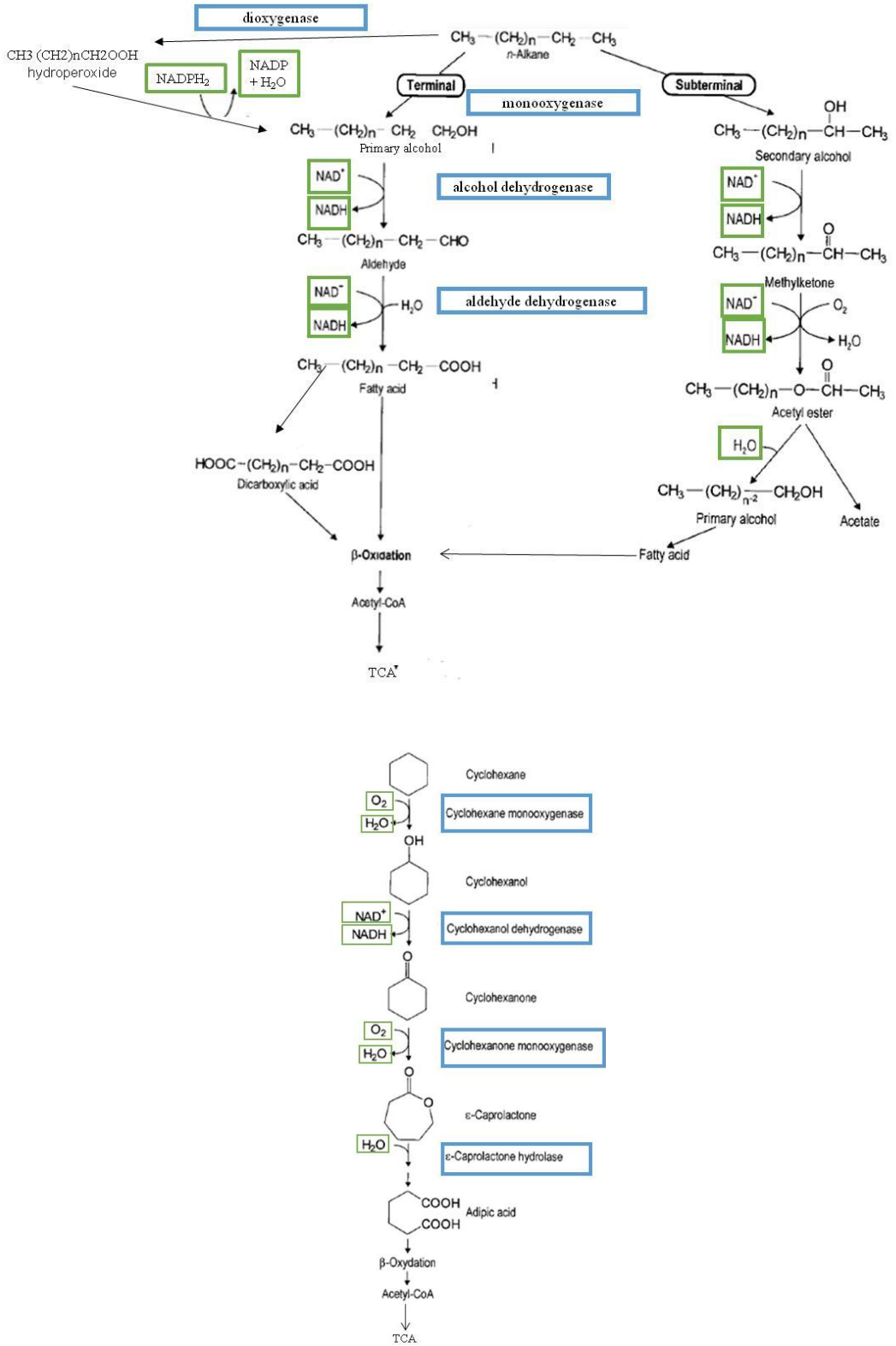


Fig. 3–Degradation pathways of alkanes and cycloalkane (adapted from Fritsche and Hofrichter 2001).

❖ Degrading Process of Aromatic Hydrocarbons

The pathways that metabolize aromatic compounds via catechol are shown in Fig.4. At the branchpoint, catechol is oxidized by either the intradiol o-cleavage, or the extradiol m-cleavage. Both ring cleavage reactions are catalyzed by specific dioxygenases (Fritsche and Hofrichter 2001). In the o-cleavage pathway, catechol 1,2-dioxygenase incorporates both atoms of O_2 into the ring-cleavage products, *cis*,*cis*-muconate. The products are cyclo-isomerized to the muconolactone and it is followed by a shift of the unstable enol-lactone. The lactone is easily hydrolysed to oxoadipate (Fuchs et al. 2011). This dicarboxylic acid is activated by transfer to CoA, followed by the thiolytic cleavage to acetyl-CoA and succinate (Fritsche and Hofrichter 2001). In the m-cleavage pathway, catechol 2,3-dioxygenase incorporates both atoms of O_2 into the ring-cleavage products 2- hydroxymuconic semialdehyde, which is metabolized by the hydrolytic enzymes to formate, acetaldehyde, and pyruvate (Fuchs et al. 2011)(Fig.4).

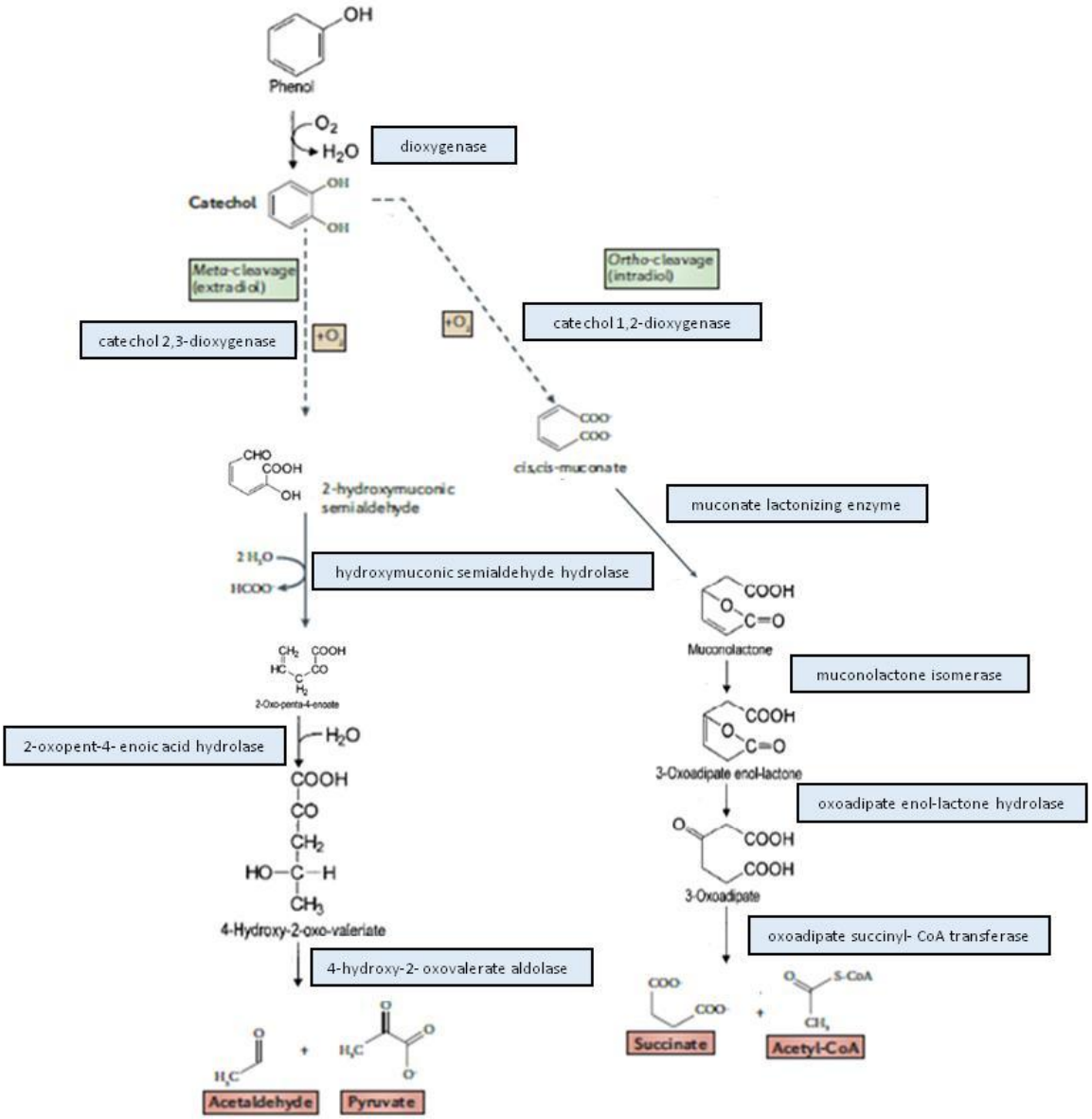


Fig. 4–The two alternative pathways of aerobic degradation of aromatic compounds: o– and m–cleavage (adapted from Fritsche and Hofrichter 2001).

In general, hydrocarbons have been ranked in the following order of decreasing susceptibility to biodegradation: n–alkanes > branched alkanes > low molecular weight aromatics > cyclic alkanes, with high molecular weight aromatics and polar compounds being extremely recalcitrant (Das and Chandran 2011).

1.5 Factors influencing petroleum hydrocarbon degradation

Theoretically, all the petroleum components can be degraded into inorganic compounds by oil-degrading microorganisms according to the mechanism of degradation. However, many studies have shown that the degradation efficiency of petroleum is different due to the complexity of various petroleum hydrocarbons and the effect of the external environmental parameters (Xue et al. 2015). There are various factors which are identified as limiting factors for the biodegradation of petroleum hydrocarbons. To make bioremediation successful, the proper knowledge of environmental parameters that influence the biodegradation of contaminant is necessary. The various factors that influence the rate of biodegradation are the following (Singh and Chandra 2014):

Bioavailability – Hydrocarbons composition and concentration influence the activity and microbial growth and hence affect the rate and extent of biodegradation (Oliveira et al. 2014).

Oxygen – Molecular oxygen is required for the occurrence of biodegradation of hydrocarbons in aerobic conditions. The concentration of oxygen has been identified as the rate limiting variable in the biodegradable of petroleum in soil. The oxygen availability in soil depends on rates of O_2 consumption by microbes, the type of soil and presence of utilizable substrates, which lead to oxygen depletion (Oliveira et al. 2014).

Temperature – At low temperature, the viscosity of oil increases while volatility of toxic low molecular weight hydrocarbons reduces, decreasing biodegradation. With the decrease in temperature, the rate of degradation also decreases because of the decreasing enzymatic activity (Gong et al. 2014).

Nutrients – Availability of limiting nutrients (N, P, K, Fe) affects microbial growth and consequently biodegradation rates. It is well established that deprivation of nitrogen and phosphorus inhibits microbial oil degradation in such ecosystems as estuaries, seawater and marine sediments, freshwater lakes, groundwater, and soils. Additional carbon source is provided by hydrocarbons in spill oil (Oliveira et al. 2014). The application of nitrogen and phosphorus following Redfield ratio is considered the most effective application of nutrients. This ratio is defined by the

atomic ratio of carbon, nitrogen and phosphorus (C:N:P = 106:16:1). Nutrients adhere to the oil facilitating microbial access.

pH – Controls microbial activity by regulating microbial metabolism. Neutral pH is favorable for biodegradation by most bacteria. Although slightly alkaline, pH provides an optimal degradation. Oil degradation is faster when the pH is between 6.5 and 8.0 (Oliveira et al. 2014).

Salinity– the rate of hydrocarbon metabolism decreased with increasing salinity in the range of 3.3 to 28.4 %, resulting in a general reduction in microbial metabolic rates (Singh and Chandra 2014).

Soil type and structure – Soil type influences the bacterial colonization and microbial activities and, subsequently, the efficiency of contaminant degradation (Oliveira et al. 2015; Gong et al. 2014). For example, coarser grain sediments allow deeper burial relatively to the fine grains. The interactions between the oil and sediments play a crucial role in the dispersion and degradation of the oil spill (Gong et al. 2014).

1.6 Aim and organization of thesis

The main objective undertaken in this thesis was to study the potential of autochthonous microorganisms from the NW Portuguese Coast, for bioremediation of hydrocarbons. The main goal was pursued through two specific objectives:

(i) Characterization of the microbial communities along the NW Portuguese Coast, regarding their potential for hydrocarbon degradation and their relation with sediment contamination; as well as the assessment of the microbial community dynamics in terms of microbial structure, bacterial richness, diversity and microbial abundance. For this study, sediments were collected in 5 sandy coastal beaches and 8 locations inside two estuaries (Douro and Minho).

(ii) Performance of microcosms experiments in laboratory conditions to assess and compare hydrocarbon degradation potential of autochthonous microorganisms for bioremediation of sediments contaminated with different types of oil, as well as alterations in bacterial richness, diversity and microbial

abundance. Sediments were collected in 3 locations along the northern Portuguese Coast: in a sandy coastal beach (Cabo do Mundo) and in Minho and Douro estuaries (Fig.5).

Fig. 5–Douro estuary (A), Minho estuary (B) and sandy coastal beach (Cabo do Mundo) (C).

This thesis is structured in four chapters. In chapter I, a general introduction is provided on accidents which have occurred by oil spill disaster, as well as on different mechanisms of oil pollution, bioremediation process, mechanisms for remediation of hydrocarbons and factors influencing petroleum hydrocarbon degradation. Chapter 2 presents the potential for hydrocarbon degradation and relation with sediment contamination. In this chapter, it is given a brief introduction about how microbial communities can be influenced by the presence of other contaminants, material and methods applied, results with respective discussion and the major conclusion about the study. Chapter 3 is about the potential of autochthonous microorganisms for removing different types of oil in microcosms. A short introduction about bioremediation process is provided followed by material and methods applied its results, discussion and main conclusions. Finally, in chapter 4, it is presented a general discussion and final conclusions.

Chapter 2

MICROBIAL COMMUNITIES ALONG THE NW PORTUGUESE COAST: POTENTIAL FOR HYDROCARBON DEGRADATION AND RELATION WITH SEDIMENT CONTAMINATION

2. Microbial communities along the NW Portuguese Coast: potential for hydrocarbon degradation and relation with sediment contamination

2.1 Introduction

Accidental oil spills have been occurring more frequently with tons lost annually via pipelines losses, as well as losses due to shipping and anthropogenic uses. The oil pollution and its derivatives are considered worldwide problems, making it an increasingly prominent concern for the environment (Malik and Ahmed 2012).

It is fundamental to bear in mind that estuarine and coastal areas are ecologically very important, with a great diversity of species, providing numerous benefits to humans. These ecosystems are undergoing anthropogenic pressure resulting from the flow of contaminants or spills or discharges from domestic and industrial wastewater (Mucha et al. 2011). This anthropogenic pressure, thus, leads to loss of biodiversity, aquatic habitat destruction and therefore, compromises the entire environment and associated ecosystem services.

Petroleum hydrocarbons are organic pollutants of major concern due to their wide persistence, distribution, complex composition and toxicity. The most common include aliphatic hydrocarbons and polycyclic aromatic hydrocarbons (PAHs) (Fuentes et al. 2014). Pollution with PAHs is of great concern due to its massive spread into the environment that is caused by its hydrophobic nature and high toxicity. Moreover, the presence of PAHs in marine and estuarine sediments provides serious risks to aquatic life and human health (Rocha et al. 2011).

Consequently, it is urgent to develop strategies to clean contaminated ecosystems in order to facilitate their recovery. Bioremediation is presented as a promising alternative to restore impacted areas. Bioremediation involves the use of biological agents, particularly microorganisms that catabolize specific molecules, destroy dangerous contaminants or transform them into less harmful forms (Fuentes et al. 2014). A combination of microbial consortia with wide enzymatic capacity is needed to remove organic contaminants (Malik and Ahmed 2012).

Several works have explored the potential of autochthonous microorganisms for bioremediation of contaminants in coastal and estuarine areas, namely metals and petroleum hydrocarbons.

For recovery of beaches affected by oil spills, Reis et al. (2014) evaluate the bioremediation potential of microorganisms from intertidal sediments of a sandy beach affected by a major oil spill demonstrating that autochthonous microorganisms were able to respond to the new oil contamination by increasing their abundance and changing the community structure. These communities presented an important potential for hydrocarbon degradation (up to 85 % for TPHs and 70 % for total PAHs), being the biodegradation stimulated by addition of an appropriate amount of nitrogen.

Almeida et al. (2013b) investigated the potential of the microbial communities present an unimpacted beach to degrade hydrocarbons. The results showed that microbial community responds to an oil spill, degrading hydrocarbons.

The application of bioremediation to oil buried beaches was then evaluated using an artificially contaminated oil layer of sand buried in a sand column subjected to tidal simulation (Pontes et al. 2013). The efficiency of biostimulation and bioaugmentation were compared to natural attenuation for 180-day. The simultaneous addition of microorganisms and nutrients at the top of the sand column were able to reach the buried oil layer and contributed to the faster oil elimination, achieving 80% of hydrocarbons degradation in 60 days (Pontes et al. 2013).

In short, the bioremediation method employing indigenous microorganisms has been accepted as an effective alternative to remove these petroleum hydrocarbons and their derivatives from contaminated soils. Therefore, for proper implementation of this technique it is necessary to have prior knowledge about the capacity of the autochthonous microbial communities to biodegrade petroleum hydrocarbons.

Microbial communities can be influenced by the presence of other contaminants that may condition the response of microorganisms to oil spills. Almeida et al. (2013a) investigated the potential effect of metals, on the biodegradation of

petroleum hydrocarbons in estuarine sediments, reporting that metals changed the microbial community structure. However, among the studied metals (Cd, Cu and Pb), only Cu displayed measurable deleterious effect on the hydrocarbons degradation process.

Mucha et al. (2013) investigated the effect of metals (Cd, Cu and Pb) on the microbial communities associated to the roots of two salt marsh plants reporting a shift in the microbial community structure, with possibly effect on the ecological function of these communities in salt marshes. Sun et al. (2012) also observed shifts in both bacterial community composition and diversity associated with sediment contaminant concentrations, particularly with metals.

This study aimed to characterize the microbial communities along the NW Portuguese Coast, regarding their potential for hydrocarbon degradation and their relation with sediment contamination. For this study, several locations were selected inside Douro and Minho estuaries and along the coastal line between these two estuaries. The Douro River estuary, throughout its watershed, receives discharges of domestic wastewater, as well as effluents from industrial and urban sources and a considerable part of domestic sewage. This estuary receives several human pressures, mainly a very high alteration of habitat, presence of physical barriers interfering with fish migratory routes, very high control of freshwater input and fisheries (Ramos et al. 2015). The Minho estuary has several tributaries, which cross agriculture, industrial and/or urban areas, consequently discharging chemical contaminants to the estuary. Although Minho estuaries present some pollution, this is considered a reference estuary in ecotoxicological studies due to relatively low human pressures (Ribeiro et al. 2015a). Coastal zone between the two estuaries is also a threatened area by potential chemical spills, due to its geographical location.

2.2 Materials and methods

2.2.1 Area of study and environmental characteristics

Water and sediment samples were collected, at low tide, along the NW Portuguese Coast from Douro estuary (D1, D2, D3, D4), Minho estuary (M1, M2, M3, M4) and at different coastal beaches (C1, C2, C4, C5, and C6) in between April and June 2014 (Fig.6 and Table 2).

Water surface samples were characterized in terms of salinity and dissolved oxygen by means of a Multi-Parameter Water Quality Sonde and were collected in sterile vials for further microbiological contamination indicators and nutrients analysis (Azevedo et al. 2013).

Sediment samples were collected and stored into sterile plastic bags. Afterwards, samples were transported to the laboratory in refrigerated ice chests. At the laboratory, a portion sediment sample was wrapped in aluminum for total petroleum hydrocarbons (TPHs) analysis and another portion was kept into sterile plastic bags for the microbial community structure analysis and both were frozen at -20°C. Remaining sediment portions were processed immediately in triplicate for total microbial abundance and hydrocarbon degraders abundance.

Table 2-GPS coordinates, along the NW Portuguese Coast, from Douro and Minho estuaries and coastal beaches.

Sampling site	GPS coordinates	Sampling site	GPS coordinates	Sampling site	GPS coordinates
D1	N 41,08.755'	M1	N 41° 52' 44,7"	C1	N 41° 49' 09,3"
	W -8,39.499'		W 008° 49' 58,7"		W 008° 52' 17,4"
D2	N 41,08.805'	M2	N 41° 53' 36,4"	C2	N 41° 44' 34,6"
	W 8,39.165		W 008° 49' 28,3"		W 008° 52' 32,9"
D3	N 41,08.404'	M3	N 41° 57' 08,2"	C4	N 41° 18' 06,2"
	W -8,36.874		W 008° 44' 43,6"		W 008° 44' 12,5"
D4	N 41,08.588	M4	N 41° 56' 02,8"	C5	N 41° 13' 12,5"
	W 8,34.679		W 008° 45' 10,0"		W 008° 42' 53,6"
				C6	N 41° 09' 08,1"
					W 008° 40' 41,8"

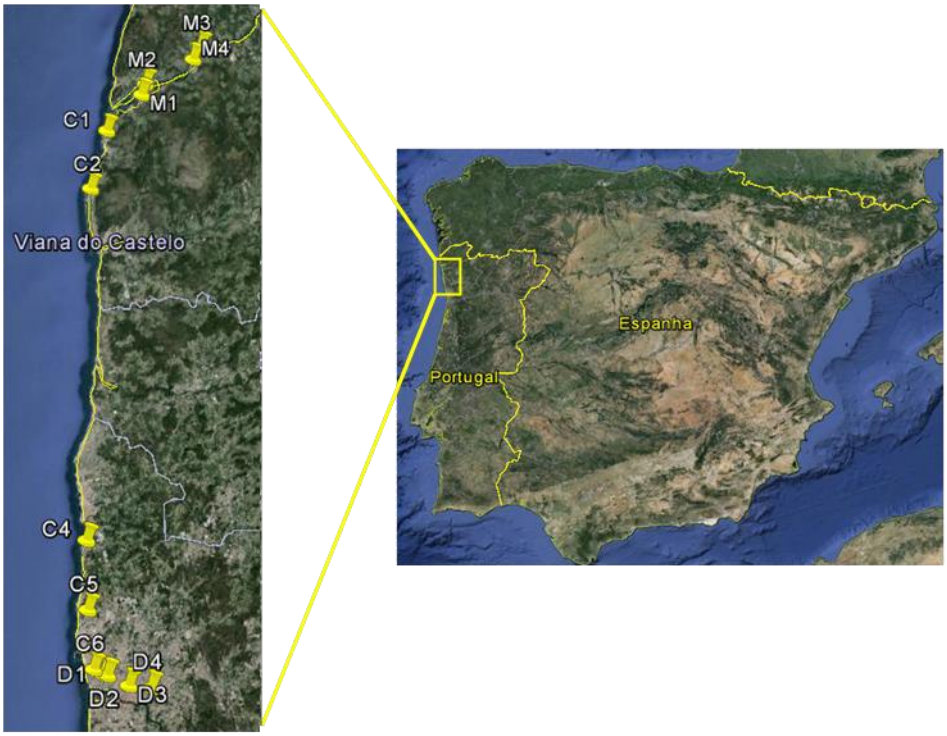


Fig. 6–Sampling locations in Minho (M1–M4) and Douro (D1–D4) estuaries and in sandy coastal beaches (C1–Vila Praia Âncora, C2–Carreço, C4–Vila Chã, C5–Cabo do Mundo and C6–Foz) (courtesy of http://earth.google.com).

2.2.2 Analytical procedures for the water samples

Dissolved orthophosphate, ammonium, nitrite were analyzed following the methods described in Grasshoff and Ehrhardt (1983). The dissolved orthophosphate (PO_4^{3-}) is typically measured by colorimetric method molybdenum blue. This method presupposes that in acidic solution, the orthophosphate reacts with ammonium molybdate and antimony potassium tartrate forming a heteropolar-phosphomolybdic acid, which is reduced by ascorbic acid to an intense blue complex.

For quantification of the concentration of ammonium (NH_3 and NH_4^+), the method is based on the fact that ammonium, in moderately alkaline solutions, reacts with the hypochlorite forming the monochloramine compound. This, in turn, in the presence of a catalyst (nitroprusside), phenol and excess of hypochlorite gives rise to an intense blue complex (indophenol).

Nitrites (NO_2^-) were quantified by the method of reaction of nitrite with an aromatic amine (sulfanilamide) to give a diazotized compound, which binds to a second aromatic amine (N- (1-naphthyl) ethylenediamine), resulting in a pink complex, whose intensity is proportional to the amount of nitrite in the solution. Nitrate (NO_3^-) was measured by an adaptation of the spongy cadmium reduction technique described in Jones (1984), subtracting nitrite value from the total. All the analyses were performed in triplicate.

Samples for fecal coliforms (FC) and fecal enterococci (FE) assessment were concentrated onto sterile membrane filters, placed respectively on mFC agar (Difco 0677-17 for FC) following a 24h incubation at 44ºC and on Slanetz & Bartley agar (Oxid CM0377,for FE) following a 48 h incubation at 37ºC (Fig.7) (Azevedo et al. 2013).

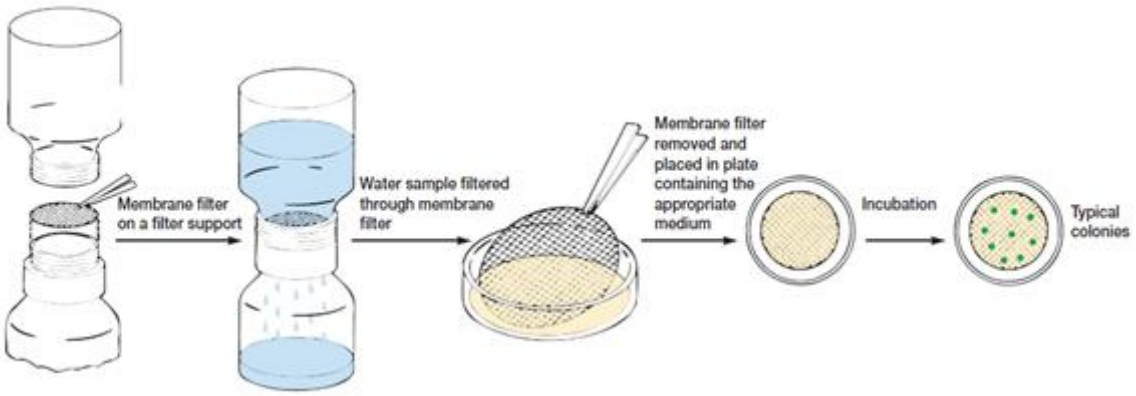


Fig. 7-The Membrane Filtration Procedure (from Prescott 2002)

2.2.3 Sediment characterization

Water and organic matter content was determined in the sediment. Sediment was dried at 100ºC to determine the water content by weights difference. For OM content, porcelain crucibles were put at 500ºC for 30 min in a muffle furnace and transferred to a desiccator to cool. After cooling, 1 g of sediment was weighted and transferred to a muffle furnace at 500 ° C for 4 hours (Fig.8A). After this time, the porcelain crucibles with samples were transferred to a desiccator to cool (Fig.8B). Finally, the porcelain crucibles were weighed to determine the OM content by weight difference.

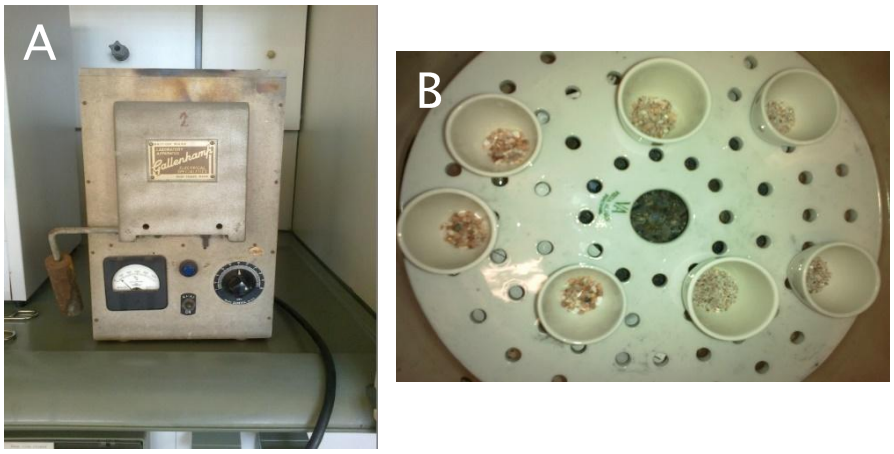


Fig. 8–A – Muffle furnace; B – Desiccator with porcelain crucibles

2.2.4 Total petroleum hydrocarbons (TPHs) determination by Fourier transform infrared spectroscopy (FT/IR) analysis

For total petroleum hydrocarbons (TPH) measurements, a previously optimized method was used (M. N. Couto et al. 2014). Briefly, c.a. 1 g of dry sediment was mixed with anhydrous sodium sulphate (1g) (1:1 (w/w)) and 10 ml of tetrachloroethylene (≥99 % spectrophotometric grade, from Sigma–Aldrich) (1:10 (w/v)) was added being followed by an ultrasonic (Elma, Transsonic 460/H model) extraction for 30 min. Next, the extracts were allowed to stand for 10 min and after that, samples were put into centrifugation for 2 min, at 1500 rpm. The obtained extracts were cleaned with desactived (0.3g) silica gel (70–230 mesh,

Machery-Nagel). This step allowed the removal of the non-mineral oil contaminants such as animal greases and vegetable oils and other polar compound, which may cause positive interferences with IR analysis. Afterwards, the extract and silica mixture were well homogenized for 10 min, and then filtrated through silanized glass wool into a disposable pipette. The obtained solution was refrigerated until analysis, usually within 1h, at 4°C. The samples extracts were analyzed by Fourier transformed infrared spectroscopy (JASCO FT/IR 460 Plus), using quartz cells of 10 mm path length (Infrasil I, Starna Scientific). Calibration standards (in tetrachoroethylene) were prepared using a stock standard solution of equal volumes of isooctane (≥99 % ACS spectrophotometric grade) and hexadecane (99 %) solutions. TPH were quantified by direct comparison with the calibration curve. The mean and respective standard deviation of three independent replicates was calculated, and the results were expressed on a dry weight basis.

2.2.5 Complementary data on sediment contamination

Complementary data on sediment contamination were obtain by the ECORISK team. PAHs and HNS were determined by SPME-GC-MS and metals by atomic absorption spectrophotometry (AAS).

2.2.6 The Total Cell Count (TCC)

For estimation of microbial abundance in sediments, the Total Cell Counts (TCC) was obtained by the 4',6'-diamidino-2-phenylindole (DAPI) direct count method (Porter and Feig 1980; Kepner and Pratt 1994). From each replicate sample it was weighed 0.25 g. Subsequently, samples were fixed with 2.5 ml formaldehyde (4% (v/v)), previously filtered (0.2 µm-filtered) and allow to rest for at least 1 hour. Hereafter, 2 drops of Tween (0.2 µm-filtered, 12.5% (v/v)) were added. Samples were stirred for 15 min, resting for 15 min followed by 10 min of ultrasonication (VWR Ultrasonic Cleaner) and maintained overnight at 4 °C. The desired volume of sample (50-500 µL) was added to test-tube. Henceforward, 2.5 mL of saline solution (0.2 µm-filtered, 9 g L^{-1} NaCl) and 2 drops of Tween (0.2 µm-filtered, 12.5% (v/v)) were added to test-tube. Samples were then stained with 10 µl DAPI

(0.5 mg ml^{-1}) and incubated in the dark for 15 min. After this time, solutions were filtered onto black Nucleopore polycarbonatefilters (0.2 µm pore size, 25 mm diameter, Whatman, UK) under gentle vacuum (Fig.9A) and washed with 5 mL of autoclaved distilled water (0.2 µm-filtered). Membranes were set up in glass slides (Fig.9B) and cells counted on an epifluorescence microscope (Leica DM6000B).

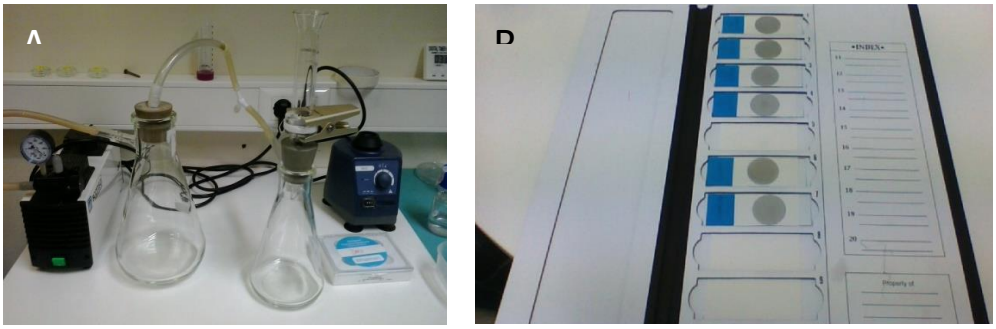


Fig. 9-A: Vacuum filtration system; B: Sets of glass slides

2.2.7 Abundance of hydrocarbon degraders

To estimate the oil degradation capacity of the indigenous microbial community, we adopted a modified most probable number (MPN) protocol (Wrenn and Venosa 1996)(Wrenn and Venosa 1996), performed in 96-well microtiter plates (12 columns x 8 lines) (Fig.10). Bushnell-Haas (BH) medium supplemented with 2% NaCl was used as the growth medium for MPN procedures (180 µL BH/well). After filling the 5*12 wells of the microtiter plates with growth medium, 10 µL per well of crude oil (pre-filtered 0.2mm) was added. For each sample, 1 g of sediment was mixed in 1 mL of BH. 180 µl from supernatant were transferred to the first well of microtiter plates and tenfold serial dilutions were performed from then on. The plates were inoculated by adding 20 µL of each dilution to five rows of 11wells. Five wells remained uninoculated to serve as sterile control. MPN plates were incubated for 2 weeks at room temperature. After incubation, 50 µL of filter sterilized Iodonitrotetrazolium violet (3 g L^{-1}) was added to each well. Positive wells were scored after overnight incubation at room temperature. The Most Probable Number Calculator V 1.00 program was used to calculate the MPN.

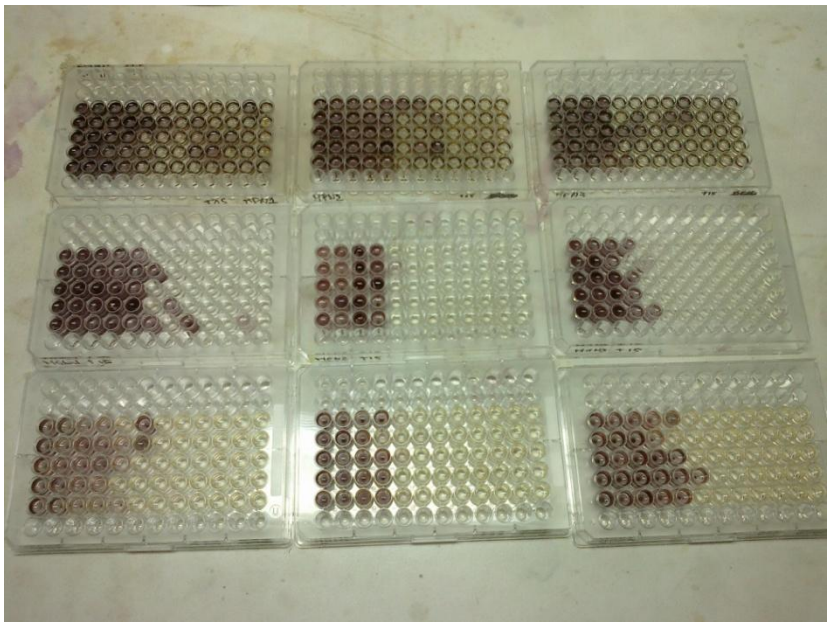


Fig. 10–Microplates for HD determination

2.2.8 DNA extraction

DNA was extracted from all sediment samples (three replicates) by Power Soil ® DNA Isolation Kit (Mo Bio Laboratories, Inc). The quality of extracted DNA was evaluated in a 1.5% electrophoresis agarose gel.

2.2.9 Bacterial community structure

Bacterial community structure was evaluated by ARISA (Automated Ribosomal Intergenic Spacer Analysis), a technique that allows amplification of the 16S-23S intergenic spacer region in the rRNA operon (Fisher and Triplett 1999). ARISA exploits the variability in the length of the intergenic spacer (IGS) between the small (16S) and large (23S) subunit rRNA genes in the rrn operon (Ranjard et al. 2001). The IGS, depending on the bacterial species, displays significant heterogeneity in both length and nucleotide sequence (fisher). ARISA has significantly contributed to advancing microbial community ecology and it is very well suited for a general overview of changes in abundant bacterial types (Gobet et al. 2014).

DNA was amplified using ITSF (5′ GTCGTAACAAGGTAGCCGTA-3′) and ITSReub (5′-GCCAAGGCATCCACC-3′) primers set (Cardinale et al. 2004), which amplifies the ITS1 region in the rRNA. These primers can be considered as the most promising primer sets for this method (Purahong et al. 2015). PCRs (polymerase chain reaction) were performed in duplicate with a total volume of 25 µL containing between 0.5 µL and 1 µL of DNA, 3× Taq PCR Buffer, 2 mM of $MgSO_4$, 0.4 µM of ITSF, 0.4 µM of ITSReub, 0.2mMof dNTPs, 1 mg mL^{-1} of bovine serum albumin (BSA) and 2.5 U of Taq DNA polymerase. The PCR program started at 94 °C for 2 min, followed by 30 cycles at 94° C for 45 s, 55 °C for 30 s and 72 °C for 2 min. Final extensions at 72 °C for 7 min. The PCR products were visualized in a 1.5 % electrophoresis agarose gel.

2.2.10 Electrophoresis agarose gel

To prepare the agarose gel, 1.5 g of agarose were mixed with 100 mL of TAE (1x) (1.5 % agarose gel) and the mixture was heated in the microwave for 4 minutes (2 minutes + 2 minutes). This time is necessary to fully dissolve the agarose. Then, 0.5 µl of SYBR® Safe was added and the gel was left to polymerize for 30 minutes. After that, the gel was placed in a horizontal electrophoresis cell (BIO RAD) and 5µl of each sample were loaded. The samples was turned on at 90 V for 30 minutes for extracted DNA and 90 V for 45 minutes for PCR products.

2.2.11 PCR products purification and quantification

PCR products were purified by UltraClean ® 15 Purification Kit (MO BIO Laboratories, Inc).

2.2.12 PCR products quantification

PCR products were quantified by Quant-it HsDNA assay kit and the Qubit fluorometer (Invitrogen). The work solution was made taking into account the following quantities: per each sample, 199 µL of Buffer and 1 µl of Qubit™ dsDNA HS reagent. The work solution was homogenized and distributed through the tubes. For the calibration, two standards were made: S1 and S2. For that, 190 µl

the work solution and 10 µl of each standard were added in the respective tubes and waited up for 2 minutes. For the samples, 198 µl of work solution and 2 µl were added and waited up for 2 minutes. First, the equipment calibration was made and then, the analyses of the samples were performed. Sample fragments were run on a ABI3730 XL genetic analyzer at STABVIDA Sequencing Facilities (Lisbon, Portugal).

2.2.13 Statistical and data analysis

The mean and standard deviation values of three replicates were calculated. Microbial enumeration data was normalized by logarithm (log10) transformation prior to statistical analysis. Differences on nutrient analysis (nitrite, nitrate, ammonium and phosphate), fecal indicator bacteria (FCs and FEs), HD abundance, TCC, TPH, PAHs, HNS and metals were analyzed by parametric one-way ANOVA (analysis of variance). Significant ($p < 0.05$) differences were detected by a multiple Tukey comparison test. All statistical tests were performed using the commercial software STATISTICA (version 12).

The multivariate method available in the Primer 6 software package (Clarke and Gorley, 2006), such as principal components analysis (PCA) was applied to the environmental abiotic data set. Measures included water and OM content, heavy metals, HNS, TPHs and total PAHs concentrations in sediments. Data were log (X+1) transformed and normalized prior to analysis to have comparable (dimensionless) scales.

Operational taxonomic units (OTUs) were analyzed by Peak Scanner™ version 1.0 Software (Applied Biosystems). Data was transferred to an excel sheet for further processing. In data analysis, fragments with Fluorescence Units below 50 were considered machine “background noise” and were not accounted for. In data analysis, fragments of less than 200 bp were removed since they were considered to be too short ITS for bacteria. Then, values corresponding to peak areas were imported into the Primer 6 software package (version 6.1.11) (Clarke and Gorley 2006). To evaluate bacterial community structure, the matrix was normalized using the presence/absence pre-treatment function and a similarity matrix was created using the Bray-Curtis similarity method that was used for hierarchical cluster analysis. Samples clustering were generated using the group average method and the Simprof test was performed to test differences between clusters

generated. The same similarity matrix was used to create a multidimensional scaling (MDS) plot using the default parameters with a minimum stress of 0.01. To analyze differences on microbial community, an analysis of similarities (ANOSIM; based on Bray–Curtis similarity) was performed. The ANOSIM is a permutation-based hypothesis statistical test, equivalent to univariate ANOVA, which tests for differences among groups (multivariate) of samples from different locations or experimental treatments (Danovaro et al. 2006). Bacterial richness and diversity index were calculated from the ARISA profiles to better address the ecological description of the bacterial community within samples. For these calculations, it was assumed that the number of peaks represented the species number (phylotype/genotype richness), and that the peak height represented the relative abundance of each bacterial species. The bacterial richness was expressed as the total number of unique OTUs (peaks) identified in each electropherogram. The Shannon–Wiener diversity index, which takes into account the number of species present and their relative importance within the assemblage, was calculated using the PRIMER software (Clarke and Gorley 2006).

To examine the relationship between (dis)similarities in community structure and (dis)similarities in environmental variables, BIO-ENV method was used. The BIO-ENV analysis enables the selection of the abiotic variable subset that maximizes the rank correlation between biotic and abiotic (dis)similarity matrices. Spearman rank coefficient (ps) was used to establish correlations between biological and abiotic parameters. The software PRIMER was used in this analysis.

2.3 Results

2.3.1 Characterization of the water

Water from the different sampling location was characterized in terms of nutrient analysis (nitrite, nitrate, ammonium and phosphate), fecal indicator bacteria (FCs and FEs), O_2 and salinity (Table 3).

Table 3–Characterization of the water from the different locations: Douro estuary (D1–D4); Minho estuary (M1–M4) and coastal beaches (C1–C6) (nd – not detected).

Sampling site	FCs (UFC 100mL^{-1})	FEs (UFC 100mL^{-1})	Phosphate (uM)	Nitrate (uM)	Nitrite (uM)	Ammonium (uM)	Salinity	O_2 (mg l^{-1})
D1	8533	200	0.95±0.08	25±1	0.40±0.09	4.3±0.6	6,50	10,07
D2	7600	100	1.3±0.2	26±2	0.5±0.1	3.9±0.4	2,88	10,24
D3	9467	1000	1.1±0.1	54±1	0.9±0.2	6.0±0.3	0,11	10,21
D4	5867	67	1.1±0.1	51±2	1.0±0.1	4.4±0.4	0,09	10,39
M1	60	40	1.6±0.2	14±2	0.3±0.2	2.4±0.8	6,50	11,40
M2	3640	1200	1.55±0.08	9±1	0.18±0.09	52±6	2,88	10,98
M3	20	20	2.7±0.1	22.3±0.4	1.07±0.05	3.2±0.2	0,11	10,93
M4	n.d	n.d	1.57±0.08	29.8±0.3	0.27±0.06	4±1	0,09	10,55
C1	n.d	n.d	0.43±0.04	0.9±0.3	0.2±0.2	27±2	32,00	10,78
C2	n.d	1	0.6±0.2	0.5±0.1	0.12±0.06	3±1	32,20	14,58
C4	n.d	n.d	1.0±0.1	1.2±0.1	0.21±0.02	1±1	33,40	14,14
C5	5	8	1.72±0.02	12.4±0.6	0.1±0.1	1.2±0.2	33,00	9,84
C6	121	3	2.45±0.05	7.5±0.6	1.0±0.4	1.6±0.5	31,30	10,67

Data from water characterization showed that the Douro estuary was the most contaminated area, presenting, in general, higher levels of fecal indicator bacteria and nitrates. Minho estuary presents the highest variability, with one of the sampling location, M2, presenting high values of fecal contamination and ammonium. In the coastal zone, same points arise with high level of nitrate (C5 and C6) and ammonium (C1) concentrations.

2.3.2 Characterization of the sediments

Sediment form the different sampling location were characterized in term of, % water, % OM and sediment type, of the different sampling sites (Table 4).

Table 4–Characterization of the sampling sediment from the different locations: Douro estuary (D1–D4); Minho estuary (M1–M4) and coastal beaches (C1–C6) (* Mean and standard deviation, n = 3).

Sampling site	% Water	% OM*	Sediment type
D1	13.58	1.38 ± 1.08	Sand
D2	43.03	9.1 ± 0.8	Mud
D3	35.82	9 ± 2	Mud
D4	42.1	9.1. ± 0.8	Mud
M1	31.25	3.8 ± 0.9	Mud
M2	24.77	3 ± 1	Mud
M3	43.8	11 ± 1	Mud
M4	22.13	3 ± 2	Sand
C1	8.53	1.74±1.22	Sand
C2	11.69	6.15±1.02	Sand
C4	5.65	2.45±1.53	Sand
C5	6.25	3.18±0.57	Sand
C6	0.88	1.78±0.53	Sand

Most of the samples collected inside the estuaries were muddy sediments, with high levels of water and OM. All the samples collected along the coast correspond to sandy sediments with low levels of water and OM.

2.3.3 Total Petroleum Hydrocarbons

Total Petroleum Hydrocarbons (TPHs) levels were lower in the sandy sediments, with values below detection limits (0.047 mg g^{-1}) in all samples from the sandy coastal beaches (Fig.11). Concerning the Douro estuary, the TPHs concentration in sediments varied between 0.047 and 0.701 mg $g^{-1}_{\text{dry sediment}}$. For Minho estuary, the values varied between 0.047 and 0.240 mg $g^{-1}_{\text{dry sediment.}}$ Thus, muddy sediment had significantly ($p<0.05$) higher levels of TPHs. In general, higher values were observed in the Douro estuary than in the Minho estuary or the sandy coastal beaches.

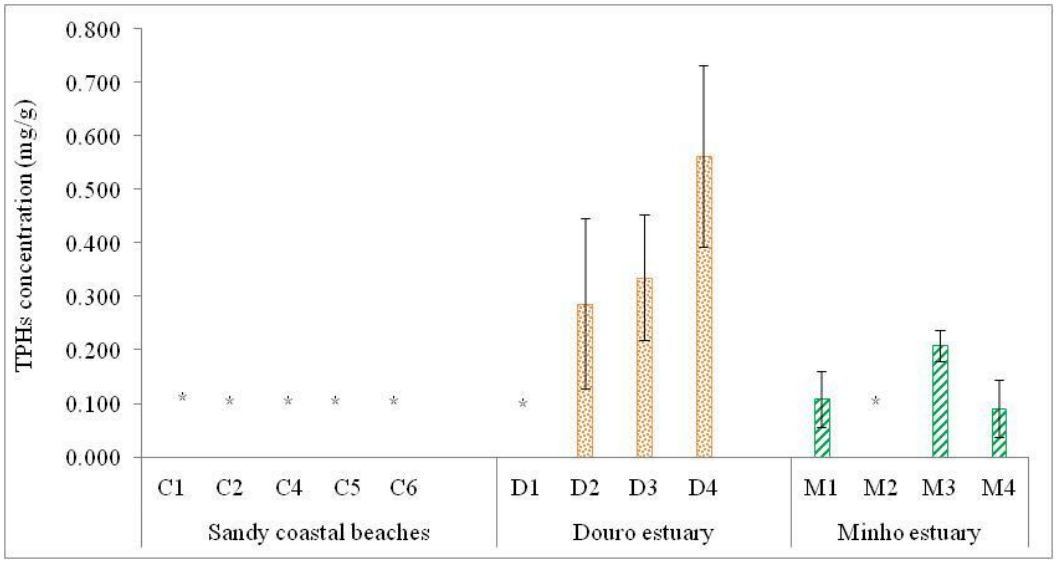


Fig. 11–Concentration of total petroleum hydrocarbons (mean and standard deviation, n = 3). *values below the limit detection (0.047 mg g^{-1})

2.3.4 PAHs, HNS, metals in sediments

The PAHs, HNS and metals were analyzed for Douro and Minho estuaries and sandy coastal beaches sediments.

Regarding PAHs (Table 5), higher values were observed for Phenanthrene, Anthracene, Fluoranthene, Pyrene and Benz(a)anthracene. Phenanthrene was the only PAHs detected in all sampling locations. High concentration of total PAHs were observed in Douro estuary, mainly in D3. Regarding the HNS (Table 6), only 4 of the analyzed compounds were detected: Toluene, m-Xylene, Chloroform, Tetrachloroethylene. Higher concentrations were observed in the coastal zone, where Chloroform and Tetrachoroethylene were detected in all the sampling sites. For metals, higher concentrations were registered in the Douro estuary, namely in D4 (Zn, Cu and Ni), D2 (Zn) and D3 (Zn) (Table 7).

Table 5–PAHs levels obtained in sediments from the different locations: Douro estuary (D1–D4); Minho estuary (M1–M4) and coastal beaches (C1–C6). (mean and standard deviation, n = 3). < d.l – values below the limit detection.

	Sampling site												
	D1	D2	D3	D4	M1	M2	M3	M4	C1	C2	C4	C5	C6
PAHs (ng g^{-1})													
Naphthalene	1.2±0.5	5.2±0.4	6.2±0.3	5.7±0.6	2.3±0.6	2.6±0.2	< d.l	4±1	0.7±0.1	0.6±0.2	1.1±0.6	0.9±0.5	0.9±0.3
Acenaphthylene	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Acenaphthene	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Fluorene	< d.l	2.9±0.5	7±3	2±1	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Phenanthrene	2.3±0.2	31±2	103±49	25±2	8±2	11.9±0.7	3.2±0.2	13±2	2.2±0.4	2.7±0.4	1.4±0.1	1.35±0.02	1.3±0.2
Anthracene	< d.l	5.8±0.5	28±15	4.5±0.6	1.0±0.3	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Fluoranthene	1.9±0.3	37±7	200±68	32±2	4.7±0.4	< d.l	< d.l	11±5	< d.l	< d.l	< d.l	< d.l	< d.l
Pyrene	2.6±0.2	34±4	188±56	35±3	5±1	6±3	2.2±0.3	11±4	< d.l	< d.l	< d.l	< d.l	< d.l
Benz(a)anthracene	3.8±0.2	16±1	98±17	15±1	7.46±0.04	7.7±0.2	7.21±0.08	9±1	< d.l	< d.l	< d.l	< d.l	< d.l
Crysene	< d.l	8±1	72±8	7.0±0.1	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Benz(b)fluoranthene	< d.l	4±2	37±7	4±1	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Benz(k)fluoranthene	< d.l	< d.l	18±4	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Benz(a)pyrene	< d.l	1.9±0.3	32±9	2.5±0.3	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Indene(1,2,3-cd)pyrene	2.0±0.6	4.7±0.2	10±1	5.9±0.1	4.9±0.2	3.1±0.4	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Dibenz(ah)anthracene	2.8±0.1	4.61±0.02	5.4±0.1	4.8±0.2	4.5±0.1	4.5±0.2	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Benz(ghi)perylene	2.7±0.5	4.5±0.3	8.8±0.7	5.0±0.3	4.6±0.1	4.1±0.3	< d.l	4.7±0.2	< d.l	< d.l	< d.l	< d.l	< d.l
Total PAHs	19±2	156±18	811±233	146±8	40±1	45±7	13.6±0.6	52±9	4.4±0.3	5.3±0.6	2.5±0.7	2.3±0.5	2.1±0.5

Table 6–HNS levels obtained in sediments from the different locations: Douro estuary (D1–D4); Minho estuary (M1–M4) and coastal beaches (C1–C6). (mean and standard deviation, n = 3). < d.l – values below the limit detection.

	Sampling site												
	D1	D2	D3	D4	M1	M2	M3	M4	C1	C2	C4	C5	C6
HNS (ng g^{-1})													
2-Propenenitrile	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
1,1-Dichloroethane	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Chloroform	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	5±1	18±2	13±2	5±1	10±2
1,1,1-Trichloroethane	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
1,2-Dichloroethane	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Benzene	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Carbon Tetrachloride	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Trichloroethylene	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
1,2-Dichloropropane	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
cis-1,3-Dichloropropene	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
trans-1,3-Dichloropropene	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Toluene	< d.l	< d.l	< d.l	0.83±0.02	< d.l	< d.l	< d.l	7±2	< d.l	< d.l	< d.l	< d.l	< d.l
1,1,2-Trichloroethane	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Tetrachloroethylene	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	23±2	18±3	13±3	15±2	18±2
Chlorobenzene	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Ethylbenzene	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
m-Xylene	< d.l	< d.l	< d.l	1.2±0.07	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
p-Xylene	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Butylacrylate	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Styrene	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
o-Xylene	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
1,3-Dichlorobenzene	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
1,4-Dichlorobenzene	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
1,2-Dichlorobenzene	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l
Cyclohexylbenzene	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l	< d.l

Table 7–Metal levels obtained in sediments from the different locations: Douro estuary (D1–D4); Minho estuary (M1–M4) and coastal beaches (C1–C6). (mean and standard deviation, n = 3). < d.l – values below the limit detection.

	Sampling site							
	D1	**D2**	**D3**	**D4**	**M1**	**M2**	**M3**	**M4**
Metals (µg g^{-1})								
Zn	18±1	87±4	97±5	108±2	44±2	40.8±0.7	20±2	87±5
Cu	1.7±0.6	9.6±0.2	16±3	22±1	3.8±0.1	4.3±0.3	1.3±0.2	7±1
Pb	5±1	3.6±0.7	3±2	6±2	0.30±0.03	< d.l	< d.l	< d.l
Ni	7±2	15±1	12±1	14±2	10±2	12±2	6.1±0.9	19±2
Cd	< d.l	0.076±0.001	0.3±0.2	0.22±0.05	< d.l	< d.l	< d.l	0.09±0.01
Hg	0.07±0.01	0.30±0.02	0.25±0.02	0.27±0.02	0.18±0.03	).193±0.00	0.074±0.001	0.159±0.006
	C1	**C2**	**C4**	**C5**	**C6**			
Zn	< d.l	5.9±0.6	4±1	< d.l	< d.l			
Cu	0.72±0.06	0.62±0.06	0.6±0.1	0.4±0.1	0.4±0.1			
Pb	0.6±0.1	1.08±0.03	< d.l	< d.l	< d.l			
Ni	0.46±0.06	1.15±0.09	0.46±0.02	0.61±0.03	0.55±0.08			
Cd	< d.l	< d.l	< d.l	< d.l	< d.l			
Hg	< d.l	0.065±0.009	< d.l	< d.l	< d.l			

2.3.5 Principal components analysis (PCA)

In order to visualize similarities between the different locations based on water characterization, PCA was applied to nitrate, nitrite, phosphate and ammonium concentrations, salinity, O_2, FCs and FEs (Fig.12). The centered log ratio procedure was used to produce a normalized data set. Data were auto scaled and log transformed before PCA. The PCA plot explained a total of 65.3 % of the data variability. PC1 (explained 41.6% of the data variability) was positively influenced by the salinity and O_2 and negatively influenced by FCs and FEs, separating locations the Douro estuary from the two other zones. PC2 (explained 23.7% of the variability in the data) was positively influenced by the Nitrite, Nitrate and Phosphate and negatively influenced by ammonium, separating most locations the Minho estuary from the two other zones.

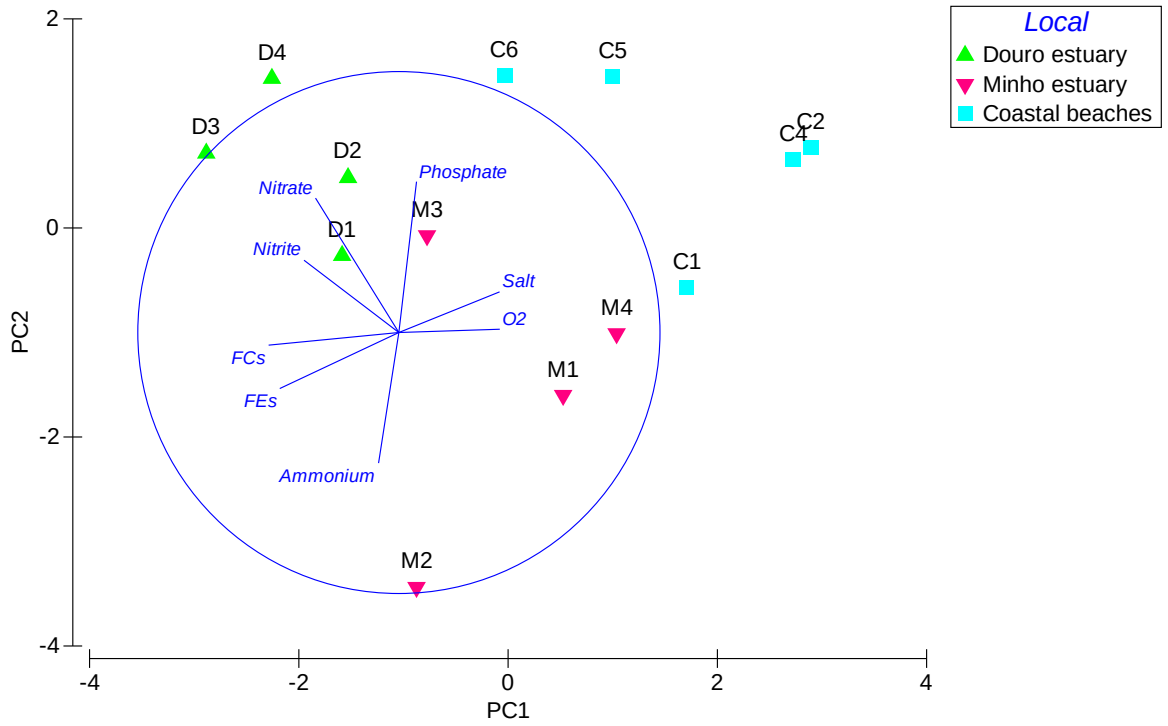


Fig. 12–Principal Component Analysis (PCA) ordination based on data from water characterization

In order to visualize similarities between the different sampling, based a sediment characterizatics. PCA was applied to % water, % OM, metals (Zn, Cu, Pb, Ni, Hg and Cd), total PAHs, HNS (chloroform, toluene, tetrachloroethylene and m-xylene) and TPH (Fig.13). The centered log ratio procedure was used to produce a normalized data set. Data were auto scaled and log transformed before PCA.

The obtained PCA plot explained a total of 80.2 % of the data variability. PC1 (explained 68.6% of the data variability) separate the different sampling areas, with Douro located in the negative extreme, corresponding to high values of most of the contaminants, and coastal beaches in the opposite extreme, corresponding to lower values of most contaminants, except Chloroform and Tetrachloroethylene. PC2 (explained 11.6% of the variability in the data) was positively influenced by % water, m-xylene, Zn, Ni, Hg, and negatively influenced by % OM, Pb, Cd, Cu, TPH, m-xylene, Tetrachloroethylene, Chloroform separate Minho locations in the upper extreme from the other locations, with D4 in the opposite extreme.

In addition, we can observe that sandy coastal beaches differ from the Douro and Minho estuaries. Considering a global vision, the Douro estuary has a higher concentration of pollutants.

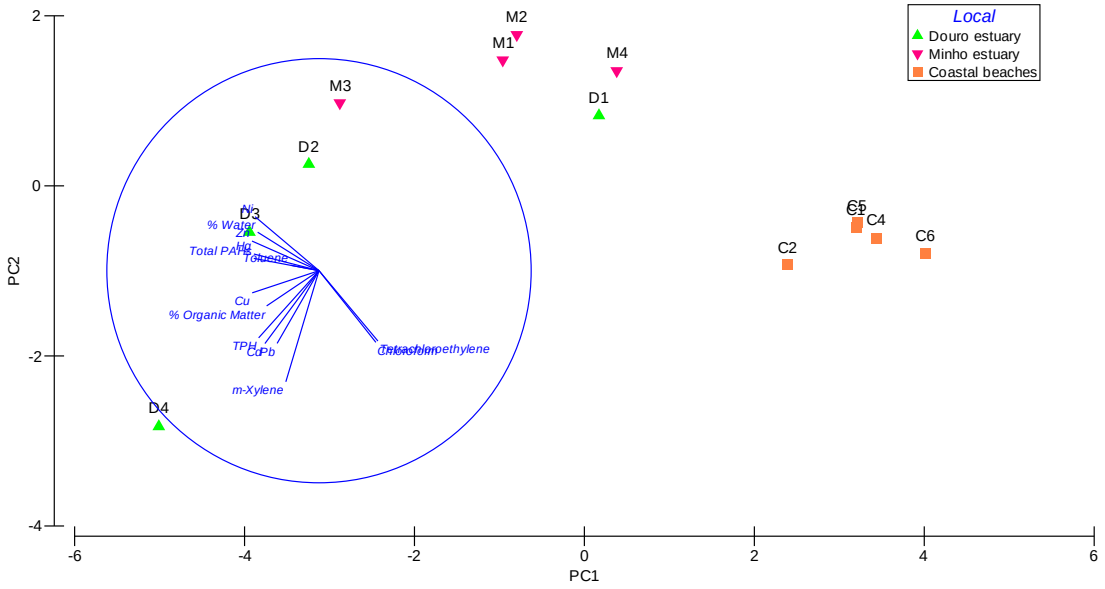


Fig. 13–Principal Component Analysis (PCA) ordination based on data from sediment characterization.

2.3.6 Microbial Abundance

The Total Cell Count (TCC) and hydrocarbon degrading microorganism (HD) abundance were estimated for sandy coastal beaches, Minho and Douro estuaries. In sediments collected from the Douro estuary, the values concerning the TCC range from 6 to 8 $\log_{10}$ cells $g^{-1}_{\text{wet sediment}}$. Regarding the HD values, the range is 3 to 5 $\log_{10}$ $g^{-1}_{\text{wet sediment}}$. It was observed that the sandy sampling location (D1) presented a lower number of total cells, having, however, a higher level of hydrocarbon degraders. The differences observed for D1 sampling point in both parameters are statistically significant ($P < 0.05$) when compared to the other sampling points held in the Douro estuary (Fig.14).

For the Minho estuary, in all sampling points, the data indicate an average value of 7 $\log_{10}$ cells $g^{-1}_{\text{wet sediment}}$ for TCC and no significant ($p<0.05$) differences were observed among samples. Regarding the hydrocarbon degraders, these have

values ranging from 2 to 4 $\log_{10}$ g^{-1} $_{\text{wet sediment,}}$ whereas the highest value refers to the sampling point M1 although differences were not significant ($p>0.05$). (Fig.14).

For sandy coastal beaches, the values concerning the TCC ranged from 5 to 7 $\log_{10}$ cells g^{-1} $_{\text{wet sediment}}$. In general, results showed significant differences ($P<0.05$) among locations. The values for HD, in all sampling points, have an average value from 1 to 2 $\log_{10}$ g^{-1} $_{\text{wet sediment}}$ without a significant variation ($p > 0.05$) (Fig.14).

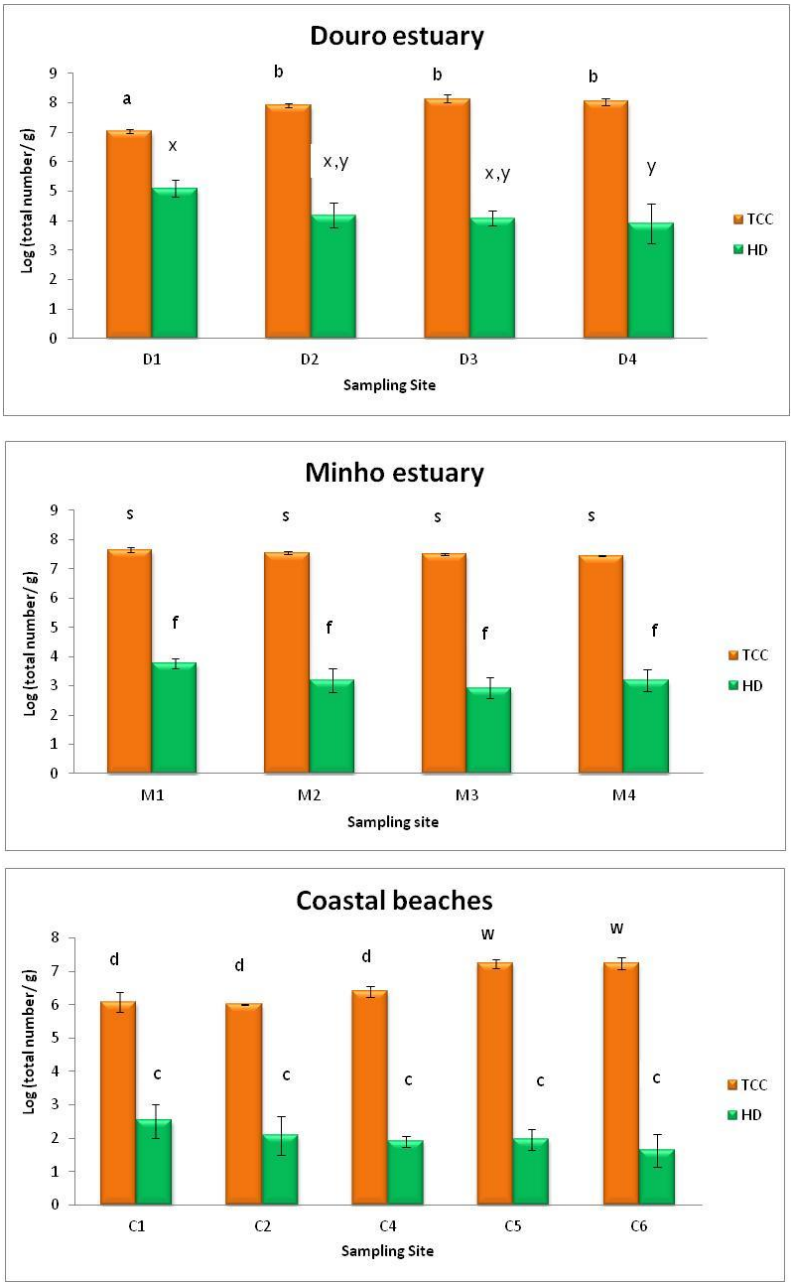


Fig. 14–TCC and HD estimated at different sampling sites (mean and standard deviation, n = 3). Different letters indicate significant ($p<0.05$) in terms of TCC (a,b,s,d,,w,) or HD (x,y, f, c).

2.3.7 Bacterial Richness and Diversity

Bacterial richness and diversity were calculated from ARISA profiles for each sediment sample. Results showed no significant differences ($p > 0.05$) in terms of bacterial richness and diversity indexes between the three zones (sandy coastal beaches, Douro and Minho estuaries). Comparing each site individually, no significant differences ($p > 0.05$) were observed in terms of bacterial richness and diversity indexes, with a single exception for diversity (D1 and D4) (Fig.15).

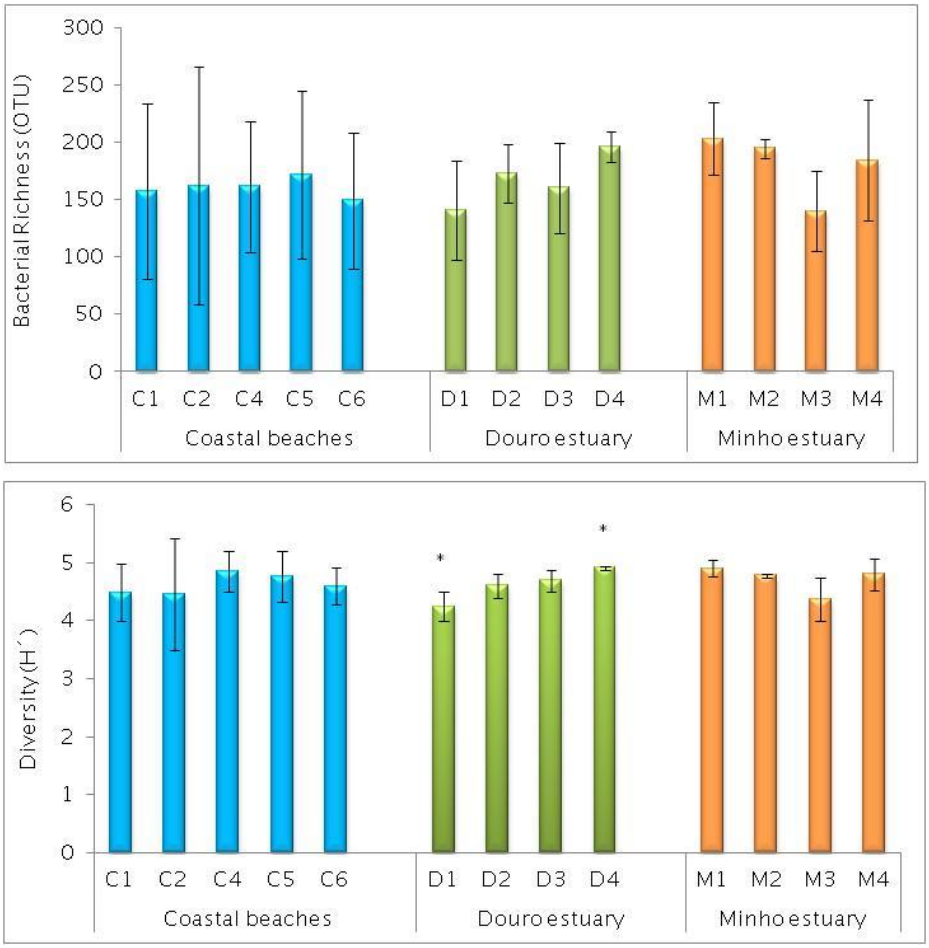


Fig. 15–Bacterial richness (A) and diversity (B) based on ARISA profiles (mean and standard deviation, n = 3). (*significantly different values (<0.05 P) when comparing D1 to D4).

2.3.8 Microbial Community Structure

ARISA analysis was performed in three replicates from each sample and ARISA fragments lengths (ALF) profiles were obtained. These fragments corresponded to total number of peaks and thus to different bacteria phylotypes. To understand bacterial community in different sampling site, MDS analysis was performed based on Bray–Curtis similarities on the presence/absence matrix obtained from ARISA analysis between samples (Fig.16). It was observed a clear differentiation between samples from the beaches and those from the estuaries. Among estuarine samples, there was a clear differentiation between Douro and Minho estuary, with the exception of samples D1 and M3. These latter samples present a sediment structure very different from the other location from the same estuary, as D1 is the only sandy sample inside the Douro estuary and present a lower organic matter content. M3 presents a much higher organic matter content than the samples from the same estuary. This point to the fact the sediment structure is affecting microbial community structure. Analysis of similarities (ANOSIM) revealed that microbial community structure was significantly ($p < 0.1$) different between the three zones (sandy coastal beaches, Douro and Minho estuaries) (Table 8), with lower R statics registered for the comparison between the two estuaries

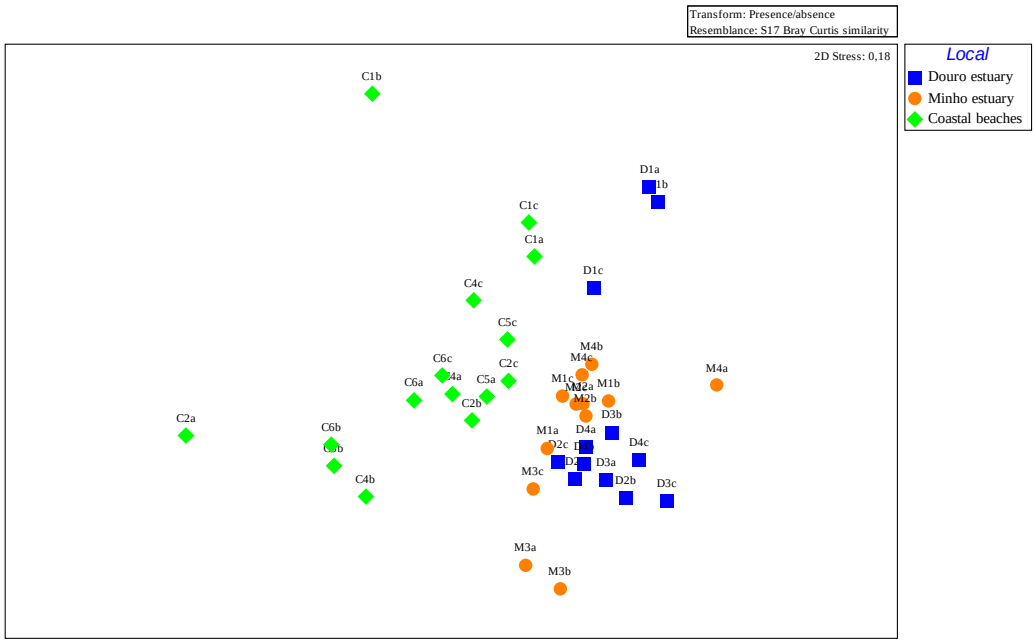


Fig. 16–MDS ordination obtained from ARISA fingerprints of bacterial communities.

Table 8–ANOSIM test for different site based on ARISA

	Statistic value (R)	Significance level (%, p>0.05)
Difference among local		
Global Test	0.378	0,1
Pairwise Tests		
Douro estuary, Minho estuary	0.309	0,1
Douro estuary, Coastal beaches	0.399	0,1
Minho estuary, Coastal beaches	0.422	0,1

2.3.9 Relationships between biological and environmental variables

BIO-ENV procedure was used to select the combinations of environmental variables that best associate the samples in a consistent manner with the microbial community structure (Table 9). The single variable that best matched biota MDS was tetrachloroethylene, followed by Ni and sediment water content. The combination of tetrachloroethylene with organic matter content constituted the overall optimum (ρs = 0.52). Although the BIO-ENV procedure does not give the direction of such correlations, it is able to indicate that these variables possibly influence the differences in community structure found between samples. Therefore, microbial communities among the studied areas appear to be affected by both contaminants and sediment structure.

Table 9–BioEnv analysis for the relationship between the microbial community and the environmental variables. Spearman rank coefficient in brackets; overall optimum is indicated in bold.

k	Best variable combinations (ρ_s)
1	Tetrachloroethylene (0.45)
2	**% Organic Matter, Tetrachloroethylene (0.52)**
3	% Water, % Organic Matter; Tetrachloroethylene (0.51)
4	% Water, % Organic Matter, Ni,Tetrachloroethylene (0.50)

2.4 Discussion

Several methodologies have been proposed for ecosystems recovery, including bioremediation. For proper implementation of this technique, it is necessary to have prior knowledge about the capacity of the autochthonous microbial communities' capacity to biodegrade petroleum hydrocarbons. The present study aimed to characterize the hydrocarbon degradation potential of autochthonous microorganisms collected along the northern Portuguese Coast with different degrees of contamination.

2.4.1 The level of pollutants along the northern Portuguese Coast

Fecal indicator bacteria, FCs and FEs, was compared to the limits imposed by European legislation for bathing waters (decreto-lei 236/98). For the FC, the Douro estuary is above the maximum admissible value (MAV) for all sampling sites. Regarding the Minho estuary, all locations are within the recommended maximum value (RMV), except M2, which is above the MAV. For sandy coastal beaches, all locations are within the RMV, except the C6. For FE, having under consideration the estuary of the Douro, D1 and D3 are above the RMV. For the Minho estuary, there is only one location (M2) above the RMV, and regarding sandy coastal beaches, all locations are within the RMV.

Azevedo et al. (2013) reported that the Lima River presented high nutrient and organic matter levels in the water column, as well as high fecal contamination. In addition, fecal contamination reached levels several times higher than the limits imposed by European legislation for bathing waters, indicating untreated wastewater discharges. This may be happening to the Douro River as well, since it has greater contamination of fecal bacteria.

Relatively to the dissolved nutrients, our results are in agreement with other authors (Azevedo et al. 2008) that showed that the river Douro was the main source of nitrate whereas in the case of phosphate and, especially, ammonium, additional sources should be considered. Ammonium highest values were recorded in August, and coincided, spatially, with increased FC bacteria. Those increased levels were probably due to wastewater discharges through tributaries.

There is a relationship between water and OM contents with sediments type. It was observed that water and OM contents in sandy sediments are more reduced, whereas for muddy sediments are highest.

Relatively to TPHs, in general, higher values were observed in the Douro estuary than in the Minho estuary or in the sandy coastal beaches.

PCA was applied for characterization of water; we can see that the Douro estuary differs from sandy coastal beaches and from Minho estuary with the exception of M3. PCA was applied for characterization of sediment; we can observe that sandy coastal beaches differ from the Douro and Minho estuaries. In a global vision, the Douro estuary has a higher concentration of metals and PAHs in the sediments.

Our results show higher concentrations for HNS in sandy coastal beaches. Although, these results cannot be compared with the literature, as no previous data was found regarding HNS contents in water or in sediment. There is only reviews, which collected information on the behavior, fate, weathering, and impact of hazardous and noxious substances (HNS) accidentally spilled at sea on the marine biota (Neuparth et al. 2011; Cunha et al. 2015). So, to our knowledge, this is the first report on HNS data in coastal water and sediments.

Our results show that there is an increase of PAHs and metals in Douro estuary compared to sandy coastal beaches and Minho estuary. This occurs because the sediment in estuaries is very fine and muddy which lead to a higher accumulation of this contaminant for adsorption. Furthermore, anthropogenic stress on the Douro River estuary results from the industrial development and, also, from the WWTP effluents that are discharged into the estuary or its tributaries. A study has reported a sewage associated input of dissolved organic carbon in the lower and middle stretches of the estuary (Magalhaes et al. 2008). The presence throughout the estuary of heavy metals (Mucha et al. 2005) and other organic pollutants such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) has already been reported in this area (Ferreira et al. 2004, 2006). Also, metal levels and PAHs concentrations normally increase with decreasing grain size (Almeida et al. 2013a).

Other studies (e.g. Zhang et al. 2002) showed that Cu concentration increases with the fine grain size and decreases as the proportion of the coarsest grain size increases. Moreover, that study shows that other metals Al, Bi, Ca, Ce, Co, Cr, and

Cs) have a similar pattern to Cu. Thus, it is shown that these elements tend to reside in the fine grains of the sediments.

2.4.2 Autochthonous microorganisms characterization along the northern Portuguese Coast

The sediment analysis allows to determine the quality of aquatic ecosystem since these are a reservoir of pollutants (Wu et al. 2014). It is well known that the characteristics of sediment, such as sediments structure (the spatial arrangement of the solid soil particles) and texture (the type and particle size), can exert an important influence and can shape the structure and the composition of microbial communities (Buyer et al. 1999; Latour 1999; Marschner et al. 2001). These characteristics influence the available surface area for microbial colonization, the microbial growth, and the ability to buffer the supply of nutrients, water availability, and a source of carbon substrate (Ribeiro et al. 2015b).

Comparing the abundance of microbial degraders of hydrocarbons in different sediments, it was observed that the Douro estuary presented a greater number of degrading microorganisms, which may be related to a previous adaptation of these communities to pollution. This observation has also been described by other authors who reported that the microbial communities from ecosystems previously contaminated with oil are better adapted to the hydrocarbon degradation due to a "memory effect", taking place when there is a previous contact with a contaminant (Almeida et al. 2013b). Thus, in an environment contaminated with oil, the proportion of hydrocarbons and degrading microorganisms increases rapidly, so the amount of these microorganisms, which have the ability to use oil as a source of carbon and energy normally, reflects the degree of contamination of the ecosystem (Almeida et al. 2013b) In addition, the Minho estuary and sandy coastal beaches also presented microorganisms with ability to degrade petroleum hydrocarbons. These results were also reported by other authors who demonstrated that the metabolic capacity requiring degrading hydrocarbons oil is widely distributed in the environment and not only in pre-contaminated sites (Almeida et al. 2013b).

It is clearly observed some relationships between HD, TCC, TPHs and some characteristics of sediments, particularly OM content. For example, sediments with higher content OM, in estuarine areas, have also higher total abundance of microorganisms (TCC). On the other hand, these same sediments present a lower abundance of degrading microorganisms (HD), despite high concentrations of TPHs. This last observation may be associated with the fact that the OM content may affect the availability of hydrocarbons preventing its degradation (Almeida et al. 2013a). They demonstrated this relationship, in which the sediments with high OM content may play an important role in adsorption, control, distribution and hydrocarbon concentration. Within Douro estuary it was observed a greater abundance of HD at the sandy sample. This result suggests that the coarser particles can provide more favorable conditions for the growth of HD. (Ribeiro et al. 2013b) also demonstrated a high density HD in aggregate corresponding to the coarser sediments, suggesting a fundamental influence of this factor.

The main purpose of the study was to characterize the potential of autochthonous microorganisms to hydrocarbon degradation collected along the northern Portuguese Coast, in which bacterial community structure was assessed. Very recent studies consider that bacterial ARISA is still a powerful tool for analyzing bacterial communities, especially for controlled experiments (Tkacz et al. 2015; Purahong et al. 2015). Gobet et al. (2013) reported ARISA has significantly contributed to advancing microbial community ecology and it is very well suited for a general overview of changes in the abundance of bacterial types.

Our results showed no significant differences ($p > 0.05$) in terms of bacterial richness and diversity indexes between the three zones (Coast, Douro and Minho). To understand the bacterial community structure, the MDS analysis was performed based on similarity between samples obtained from ARISA analysis. It was observed a clear differentiation of the microbial community structure with location confirmed by analysis of similarities (two-way crossed ANOSIM). The sites were grouped by their characteristics. These data suggest that sediment characteristics probably contributed for the dissimilarity between microbial community structures. Fine sediments provide greater surface area for microbial colonization comparatively to coarse sediments, as well as to increased stabilization of microbial cells (H. Ribeiro et al. 2013b). In order to analyze the relationships between environmental and biological variables, the BIO-ENV

analysis was performed. BIO-ENV procedure enables the selection of the abiotic variable subset that maximizes the rank correlation between biotic and abiotic (dis)similarity matrices. This analysis indicated that both the sediment structure (e.g. organic matter content) and contamination (e.g. Tetrachloroethylene) are affecting microbial community structure along the studied areas. Other studies already referred that there are specific characteristics of the sediment that may contribute with a prominent influence on microbial communities, shaping the structure and composition of these communities (H. Ribeiro et al. 2015b).

2.5 Conclusion

Pollutants are accumulated in confined areas where the sediment is fine and rich in organic matter. In these areas, the presence of organic matter contributes to the adsorption of metals and hydrocarbons. In fact, both sediment structure and contamination are shaping the microbial community structure along the NW Portuguese Coast. Furthermore, the results of the present work show that higher abundance of hydrocarbon degrading microorganisms was detected in the Douro estuary, which was also the location with higher levels of total petroleum hydrocarbons. Nevertheless, hydrocarbon degrading microorganisms were found in all collected sediments, despite the different degrees of petroleum hydrocarbon contamination. Therefore, at the selected sites, the characterized autochthonous microbial communities have the potential to degrade hydrocarbons, being important to assess experimentally their ability to bioremediation of these pollutants.

Chapter 3

BIOREMEDIATION OF DIFFERENT TYPES OF OIL IN ESTUARINE AND COASTAL ENVIRONMENTS – THE ROLE OF AUTOCHTHONOUS MICROORGANISMS

3. Bioremediation of different types of oil in estuarine and coastal environments – the role of autochthonous microorganisms

3.1 Introduction

The need for remediating polluted areas has paved the way for development of new technologies to detoxify contaminants not only through chemical or physical methods, but through biological techniques as well. Bioremediation is a set of technologies that make the removal of contaminants possible, or failing that, make a number of contaminants less harmful by means of biological activity (Mhatre and Kunde 2014).

Large amounts of crude oil have been entering the world's oceans for millions of years, and a diverse group of microorganisms has evolved to take advantage of this rich source of reduced carbon. Much of the oil comes from natural seeps, and while some adheres to sediment particles and is degraded on the sea bottom, some rises to the sea surface to form slicks, and some disperses as small droplets in the water column (Prince et al. 2013). Crude oil and derivatives are hydrocarbon mixtures, so their removal depends on their bioavailability, which is directly related to composition, chain length, branching, and steric and electronic effects.

Crude oil can be refined to produce usable products such as diesel oil, turbine oil and various forms of petrochemicals. Diesel oil is a medium distillate of petroleum composed mainly of linear chain alkanes. However, branch chain hydrocarbons and aromatic compounds are also presented (de Souza Pereira Silva et al. 2015). Turbine oil is also a petroleum derivative in which its extension contamination is low but it remains and accumulates in the environment for long periods of time due to their low biodegradability (Hosokawa, et al. 2010).

It is well known that there is no single microbial species with metabolic capacity to degrade all components of oil, and therefore its derivatives require the action of microbial consortia. A microbial consortium provides a greater spectrum of enzyme activity in bio-removal since it involves metabolic expression of microorganisms. A few factors that may limit bio-removal of pollutants are

mainly microorganisms, nutrients, pH, and temperature. Thus, the combined action of added nutrients with a consortium of microorganisms provides good results in removing hydrocarbons (Silva et al. 2015). It has been well documented that the addition of nitrogen and phosphorus significantly enhances the growth of hydrocarbon-degrading bacteria, with consequent stimulation of metabolic processes involved in oil biodegradation (Hassanshahian et al. 2014). Therefore, the knowledge of the hydrocarbon degradation potential in contaminated soil is crucial for the management of soils for bioremediation (Yang et al. 2014).

Thus, the presence of petroleum hydrocarbons on the environment causes a strong indigenous microorganisms selection that are the primary catalysts for numerous organic reactions essential to maintain the ecosystem balance. Moreover, the microbial community must be able to adjust to new biochemical pathways for contaminant incorporation (Cruz et al. 2014). Pre-exposure and subsequent re-exposure of contaminant leads to an increase in microorganism metabolic potential. A previously exposition to contamination in the microbiota leads to a biological memory that allows a major efficiency in removal of contaminants in case of continuous strokes (Megharaj et al. 2011).

Several works have explored the potential of autochthonous microorganisms for bioremediation of petroleum hydrocarbons and its derivates. Horel et al. (2012) determined the microbial community responses to exposure to crude oil and conventional diesel in a sandy beach environment. Biodegradation was assessed in mesocosm experiments with differing fuel amounts and with or without inorganic nutrient amendment. They report that hydrocarbon degradation by extant microbial populations in sandy beach environments can be stimulated and enhanced by inorganic nutrient addition.

Nikolopoulou et al. (2013) examined the effectiveness of strategies combining autochthonous bioaugmentation with biostimulation for successful remediation of polluted marine environments. They report that the use of biostimulation additives in combination with naturally pre-adapted hydrocarbon-degrading consortia (bioaugmentation) has proved to be an effective treatment and is a promising strategy that could be applied specifically when an oil spill approaches near a shore line.

Cruz et al. (2014) investigated the biodegradation of contaminated soil with biodiesel, diesel, and petroleum by autochthonous soil microorganisms and the results revealed that the autochthonous microorganisms in the soil were capable of degrading the pollutant.

In short, the bioremediation method employing indigenous microorganisms has been an effective alternative to remove these petroleum hydrocarbons and their derivatives from contaminated soils.

Several works have explored the potential of autochthonous microorganisms for bioremediation of contaminants in coastal and estuarine areas. For recovery of beaches affected by oil spills, Reis et al. (2014) evaluated the bioremediation potential of microorganisms from intertidal sediments of a sandy beach affected by a major oil spill. Results showed that autochthonous microorganisms were able to respond to the new oil contamination by increasing their abundance and changing the community structure. Almeida et al. (2013b) investigated the potential of the microbial communities presented in the intertidal zone of an unimpacted beach to degrade hydrocarbons. Results showed that the microbial community of this unimpacted beach sediment could respond to an oil spill, degrading hydrocarbons.

Our results of previous work (Chapter 2) showed that hydrocarbon degrading microorganisms were found in all collected sediments, despite the different degrees of petroleum hydrocarbon contamination. Therefore, the characterized autochthonous microbial communities have the potential to degrade hydrocarbons. Thereafter, three sites were selected to evaluated autochthonous hydrocarbon degrading microorganisms ability for the bioremediation of different types of hydrocarbon contamination.

The present work aims to study the potential role of autochthonous microorganisms for bioremediation of sediments contaminated with different types of oil (crude oil, diesel oil and turbine oil). Sediments were collected in 3 locations along the northern Portuguese Coast: in a sandy coastal beach and in Minho and Douro estuaries. We choose the Douro estuary because throughout its watershed, it receives discharges of domestic wastewater, as well as effluents from industrial and urban sources and a considerable part of domestic sewage (Ramos et al. 2015) and also due to maritime traffic. Minho estuary was chosen because this is considered a reference estuary in ecotoxicological studies

(Ribeiro, et al. 2015a). Cabo do Mundo (Matosinhos, Porto) was chosen as a sandy coastal beach because it is located near an oil refinery.

3.2 Materials and methods

3.2.1 Field of study and sediment sampling

Sediment samples were collected in February of 2015 on the beach Cabo do Mundo, in Matosinhos [41º 13´12.5´´N; 08º 42´53.6´´W], Douro River estuary [41º 8´880´´N; 08º 39´141´´W] and Minho River estuary [41º 52´039´´N; 08º 51´590´´W] (Fig.17).

Water surface samples were characterized in situ, in terms of salinity and dissolved oxygen by means of a Multi-Parameter Water Quality Sonde. In addition, water samples were collected in sterile vials for further analysis of nutrients and fecal indicator bacteria.

Afterwards, samples were transported to the laboratory under dark conditions and in refrigerated ice chests. At the laboratory, a portion sediment sample was wrapped in aluminum for total petroleum hydrocarbons (TPHs) analysis, and another portion was kept into plastic bags for the microbial community structure analysis and both were frozen at -20°C. A portion of sediment sample was used for estimation of abundance of hydrocarbon degrading microorganisms (HD) that was performed immediately by the Most Probable Number (MPN) method. Another portion of the sediment was fixed with formaldehyde and were stored in triplicate at 4°C for further analysis of microbial abundance. Remaining portions of the sediment were stored at 4°C for additional procedures, such as water and organic matter (OM) content.

Fig. 17–Sediments were collected in 3 locations along the northern Portuguese Coast: coastal beach (Cabo do Mundo) and in Minho and Douro estuaries.

3.2.2 Analytical procedures for the water samples

Dissolved nutrients (ammonium, nitrate, nitrite, phosphate) and fecal indicator bacteria (FE and FC) were analyzed as described in chapter 2 – Material and Methods – section 2.2.

3.2.3 Sediment characterization

Water and organic matter content were determined in the sediment, as described in chapter 2– Material and Methods – section 2.3.

3.2.4 Bioremediation experiments

For evaluating microbial degradation of hydrocarbons, 10 ml of homogenized sediment were placed in 50 ml flasks (microcosms) and mixed with 20 ml of Bushnell–Haas (BH) (supplemented with 2% NaCl and 20 mM NO_3^-, in KNO_3 form) , a medium usually used for the evaluation of hydrocarbon degradation by microorganisms. Although BH is already a mineral salt enrichment medium, nitrogen was added to avoid N limitation. Each flask had different treatments (crude oil, turbine oil and diesel oil) (three replicates per treatment). The different type oils were used as the hydrocarbon source. Microcosms with different oil types had a sediment/medium/oil proportion of 10:20:0.5 (v/v/v). At the beginning of the experiment, triplicate sediment samples were collected for analysis of total petroleum hydrocarbon (TPHs) and considered as T0 samples. The remaining microcosms, with triplicate sediment samples, were incubated at room temperature in the dark in a mechanical stirring at 100 rpm. This process aims at simulate the mixture of oil with water and sediment during an oil spill. The microcosms were also manually shaken once every day to improve blending between oil and sediment. Control treatment was prepared with sediment samples, in the same condition but not spiked with oil. The experiment was carried out for 15 days (Fig.18).

After 15 days of incubation, the sediment samples were removed for TPH and microbial community characterization and frozen at -20°C. A portion of sediment was removed for estimation HD ´abundances that was performed immediately by method MPN. Remaining portions of the sediment were fixed with formaldehyde and were stored in triplicate at 4°C for further analysis of microbial abundance. For hydrocarbon analysis all samples were lyophilized.

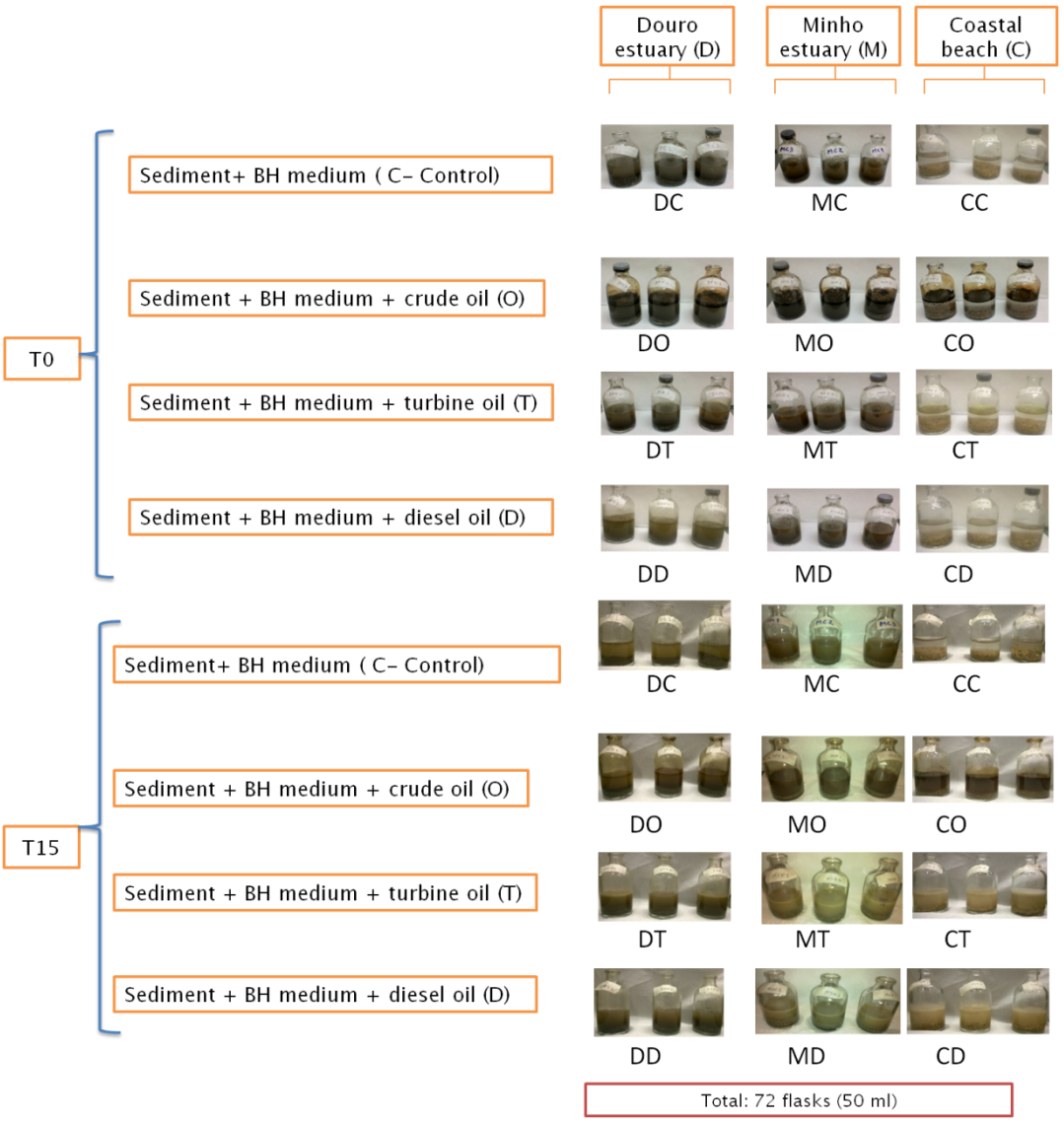


Fig. 18–Experimental design.

3.2.5 Isolation of microbial strains

Remaining solutions in contact with the respective sediments at the end of the experiment were used to perform isolation of microorganisms.

For that, a mixture of cells is spread out on an agar surface so that every cell grows into a completely separate colony, a macroscopically visible growth or

cluster of microorganisms on a solid medium, each colony representing a pure culture.

The procedure for the isolation of colonies is the following; the original sample is diluted (saline solution 0.85%) several times (Fig.19) to reduce the microbial population sufficiently to obtain separate colonies when plating. 100 µl of dilute microbial mixture is transferred to the center of an agar plate (Plate Count Agar – suspend 23,5g in 1 liter of distilled water, heat until completely dissolved and autoclave at 121ºC for 15 min) and spread evenly over the surface with a sterile bent-glass rod. The dispersed cells develop into isolated colonies then the plates were placed in an incubator until obtaining colonies. After, the more isolated colonies are pricked and transferred to the edge of an agar plate with an inoculating loop and then streaked out over the surface in one of several patterns (Fig.20). At some point in the process, single cells drop from the loop as it is rubbed along the agar surface and develop into separate colonies. Isolated cells grow into colonies and can be used to establish pure cultures (Prescott 2002). Then, pure cultures were preserved in glycerol (in eppendorfs (containing saline solution 0.85% + glycerol 17%)) and kept at -80 ° C.

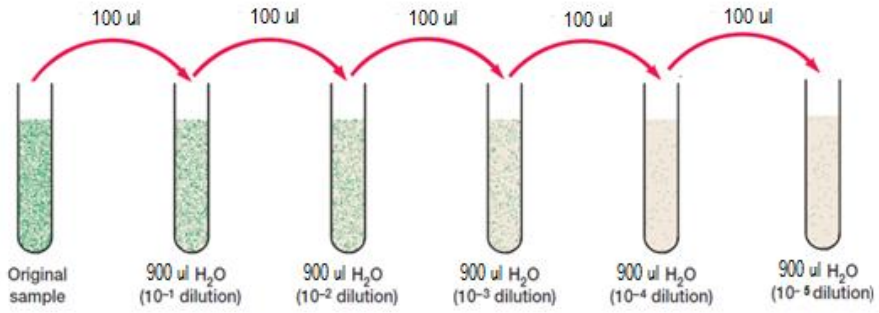


Fig. 19–Scheme for the dilution of samples (adapted from Prescott 2002).

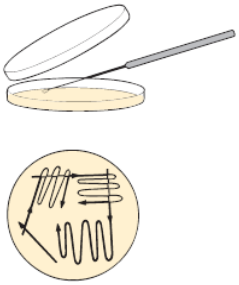


Fig. 20–Scheme used for pricking out (from Prescott 2002).

3.2.6 Total petroleum hydrocarbons (TPHs) determination by Fourier transform infrared spectroscopy (FT/IR) analysis

Total petroleum hydrocarbons (TPH) were quantified as described in chapter 2 – Material and Methods – section 2.4.

3.2.7 Total Cell Count (TCC)

The microbial abundance was determined as described in chapter 2 – Material and Methods – section 2.5.

3.2.8 Abundance of hydrocarbon degraders

HD abundance was determined as described in chapter 2 – Material and Methods – section 2.6.

3.2.9 DNA extraction

DNA was extracted from all sediment samples (three replicates) by Power Soil ® DNA Isolation Kit (Mo Bio Laboratories, Inc). The quality of extracted DNA was evaluated in a 1.5% electrophoresis agarose gel (Chapter 2 – Material and Methods – section 2.2.12).

3.2.10 Bacterial community structure

Bacterial community structure was evaluated by ARISA (Automated Ribosomal Intergenic Spacer Analysis), as described in Chapter 2– Material and Methods – section 2.2.11.

3.2.11 PCR products quantification and purification

The purification and quantification were the same described in detail in Material and Methods – section methods chapter 2 – 2.2.13 and 2.2.14, respectively.

3.2.12 Statistical and data analysis

The mean and standard deviation values of three replicates were calculated. Microbial enumeration data were normalized by logarithm (log10) transformation prior to statistical analysis. Differences on nutrient analysis (nitrite, nitrate, ammonium and phosphate), fecal indicator bacteria (FCs and FEs), HD, TCC and TPH were analyzed by parametric one-way ANOVA (analysis of variance). Significant ($p < 0.05$) differences were detected by a multiple Tukey comparison test in software STATISTICA. All statistical tests were performed using the commercial software STATISTICA (version 12).

Operational taxonomic units (OTUs) were analyzed by Peak Scanner™ version 1.0 Software (Applied Biosystems). Data was transferred to an excel sheet for further processing. In data analysis, fragments with Fluorescence Units below 50 were considered machine "background noise" and were not accounted for. In data analysis, fragments of less than 200 bp were removed since they were considered to be too short ITS for bacteria. Then, values corresponding to peak areas were imported into the Primer 6 software package (version 6.1.11) (Clarke and Gorley 2006). To evaluate bacterial community structure, the matrix was normalized using the presence/absence pretreatment function and a similarity matrix was created using tht Bray-Curtis similarity method that was used for hierarchical cluster analysis. Samples clustering were generated using the group average method and the Simprof test was performed to test differences between clusters generated. The same similarity matrix was used to create a multidimensional scaling (MDS) plot using the default parameters with a minimum stress of 0.01. To analyze differences on microbial community, an analysis of similarities (ANOSIM; based on Bray-Curtis similarity) was performed. The ANOSIM is a permutation-based hypothesis statistical test, equivalent to univariate ANOVA, which tests for differences among groups (multivariate) of samples from different locations or experimental treatments (Danovaro et al. 2006). Bacterial richness and diversity index were calculated from the ARISA profiles to better address the

ecological description of the bacterial community within samples. For these calculations, it was assumed that the number of peaks represented the species number (phylotype/genotype richness), and that the peak height represented the relative abundance of each bacterial species. The bacterial richness was expressed as the total number of unique OTUs (peaks) identified in each electropherogram. The Shannon–Wiener diversity index, which takes into account the number of species present and their relative importance within the assemblage, was calculated using the PRIMER software (Clarke and Gorley 2006).

3.3 Results

3.3.1 Water characterization

Water collected in the different sampling sites was characterized in terms of salinity, nutrient analysis (nitrite, nitrate, ammonium and phosphate) and fecal indicator bacteria (FCs and FEs) (Table 10).

Table 10–Water characterization the different sampling sites (*<d.l – values below the limit detection (0.25(μM))).

	Douro estury	Minho estary	Coastal beach
FCs (UFC 100mL^{-1})	350	360	8
FEs (UFC 100mL^{-1})	460	53	11
Phosphate (uM)	0.65±0.08	0.6±0.1	0.8±0.0
Nitrate (uM)	47±4	31±3	24±4
Nitrite (uM)	<d.l*	<d.l*	<d.l*
Ammonium (uM)	14.8±0.7	7.98±0.07	11.6±0.7
Salinity (ppt)	36	0.5	33.2

All samples were collected at low tide, but only the location from Minho estuary was under the influence of fresh water. Regarding fecal indicator bacteria, Douro and Minho estuaries contain a large number of FCs and Douro estuary presented greatest numbers of FEs. Relatively to the analysis of nutrients, nitrite concentration presented values below detection limits (0.25 μM) in all sampling sites. Douro estuary presented larger nutrient concentration with phosphate exception.

3.3.2 Sediment characterization

We can observe, as expected, the sample with the highest level is the OM content, which has the highest water content. Minho estuary presented highest water and OM content and Coast presented low water and OM content. It is noteworthy that for all sampling sites, the sediment collected is of sand (Table 11).

Table 11–Sediment type and water and OM contents in the different sampling site (*mean and standard deviation, n=3).

Local	Sediment		
	Sediment type	% Water	% OM*
Douro estuary	Sand	16.95	2.7±0.5
Minho estuary	Sand	24.13	3.8±0.7
Coastal beach	Sand	12.66	1.94±0.09

3.3.3 Total Cell Count (TCC)

Total cell counts (TCC) were estimated in sediment collected in the sampling sites (initial sediment) and in sediment from each treatment at the end of the experiment. There are no significant differences in TCC sediment between the different sampling sites. After 15 days of exposure to the different types of oil, Douro estuary only presented significant differences between initial sediment and DO. Regarding Minho estuary, significant differences were observed ($p < 0.05$) when comparing each treatment with the respective initial sediment and when comparing each treatment with the respective control. Regarding Coastal beach, significant differences were observed ($p < 0.05$) when comparing each treatment with the respective initial sediment and when comparing each treatment with the respective control. We can observe that exist significant differences between control and sediment initial (Fig.21).

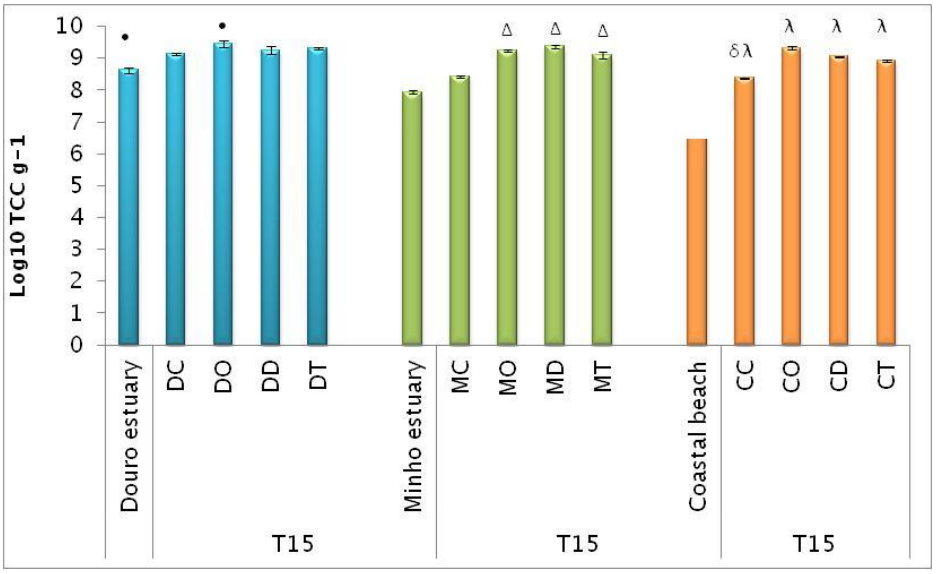


Fig. 21– Microbial abundance (mean and standard deviation, n=3) in sediments from initial sediments (Douro estuary, Minho estuary and Coastal beach) and after 15 days of experiment (T15). First letter, D–Douro; M–Minho; C–Coast and second letter, C–Control; O–Crude oil; D–Diesel oil; T–Turbine oil. •significant differences between Douro (initial sediment) and DO ($p < 0.05$); Δsignificant differences were observed ($p< 0.05$) when comparing each treatment with the respective initial sediment and when comparing with the respective control.; λsignificant differences were observed ($p< 0.05$) when comparing each treatment with the respective initial sediment and when comparing with the respective control. δsignificant differences between control and sediment initial.

3.3.4 Hydrocarbon degrading microbial abundance

In general, significant differences ($P<0.05$) in terms of hydrocarbon degrading microorganisms were observed between the initial sediment and sediments collected at the end of the experiment (Fig.22).

At the beginning of the experiment, significant differences among HD were observed, with lower abundances in Coastal beach and higher abundances in the Douro estuary. Nevertheless, after 15 days of exposure to the different types of oil, all microbial communities increased their HD and no significant differences ($P>0.05$) were observed only between Minho estuary and Coastal beach.

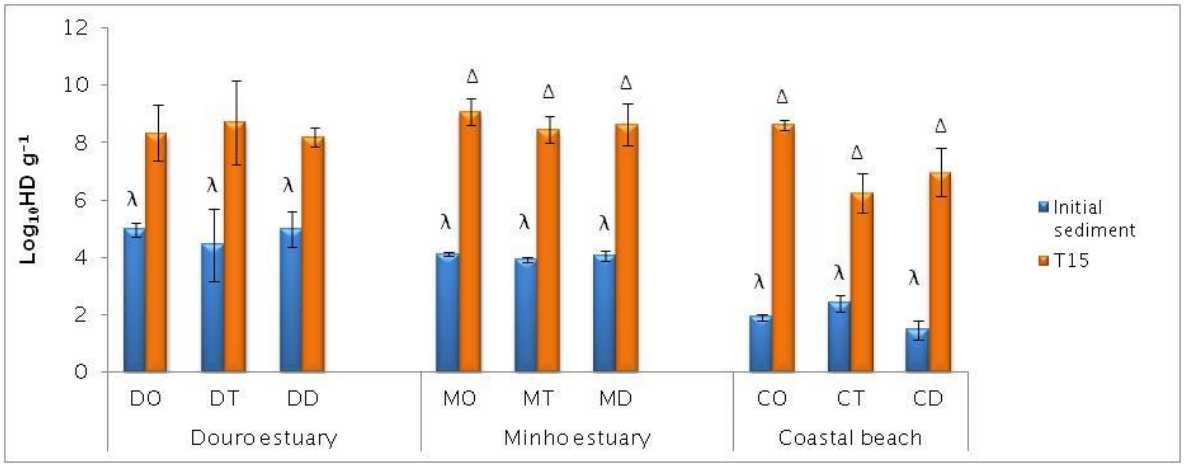


Fig. 22- Microbial abundance of hydrocarbon degraders (mean and standard deviation, n=3) in sediments from initial sediments (Douro estuary, Minho estuary and Coastal beach) and after 15 days of experiment (T15). First letter, D-Douro; M-Minho; C-Coast and second letter, C-Control; O-Crude oil; D-Diesel oil; T-Turbine oil. λ significant differences between sampling sites in the initial sediment ($p < 0.05$); Δsignificant differences between Minho estuary and Coastal beach in T15 ($p < 0.05$).

3.3.5 Potential for hydrocarbon bioremediation in sediments

To assess hydrocarbons degradation, TPH concentrations were determined at the beginning and at the end of the experience in sediment samples of all microcosms (Table 12).

For Douro and Minho estuary and Coast in general, in all treatments, significant differences ($P<0.05$) between T0 and T15 THP concentrations were observed.

Sediments from Minho estuary and Coastal beach presented the higher percentage of hydrocarbon degradation. For Douro estuary sediments, diesel oil treatment yielded the higher hydrocarbon degradation and turbine oil treatment presented lower hydrocarbon degradation. For Minho estuary sediments, crude oil treatment yielded the higher hydrocarbon degradation and diesel oil treatment presented lower hydrocarbon degradation. For Coastal beach sediments, diesel oil treatment yielded the higher hydrocarbon degradation and turbine oil treatment presented lower hydrocarbon degradation. For all sampling sites, the hydrocarbon degradation varied with the type of compound.

Table 12- Total petroleum hydrocarbons (TPH) concentrations (mg g−1, mean and standard deviation, n=3) at the beginning of the experiment (T0) and after 15 days (T15) in sediments contaminated with different types of oils, as well as the respective TPH degradation percentages (*significant (P<0.05) differences between T0 and T15 samples).

	Crude oil			Diesel oil		
Sampling Site	T0 TPH concentration (mg g^{-1})	T15 TPH concentration (mg g^{-1})	Degradation (%)	T0 TPH concentration (mg g^{-1})	T15 TPH concentration (mg g^{-1})	Degradation (%)
Douro estuary	11±1	7±3	30*	32±2	8±1	76*
Minho estuary	21±1	2.3±0.5	89*	7.6±0.5	2.0±0.6	73*
Coastal beach	8.8±0.8	1.6±0.1	81*	31±2	0.47±0.07	98*
	Turbine oil					
	T0 TPH concentration (mg g^{-1})	T15 TPH concentration (mg g^{-1})	Degradation (%)			
Douro estuary	19.6±1.9	17±1	14*			
Minho estuary	17.6±1.1	2.5±0.4	86*			
Coastal beach	20.0±0.4	5.2±0.7	74*			

3.3.6 Microorganisms isolated from hydrocarbon bioremedation experiments

After hydrocarbons bioremediation experiment, the microbial diversity and abundance of the different degrading cultures was analyzed. The results showed that for Douro estuary, 5 bacterial species were isolated from the consortium contaminated with crude oil, 3 bacterial species from diesel oil and 4 bacterial species from turbine oil. For Minho estuary, 4 bacterial species were isolated from the consortium contaminated with crude oil, 6 bacterial species from diesel oil and 6 bacterial species from turbine oil. For Coastal beach, 5 bacterial species were isolated from the consortium contaminated with crude oil, 4 bacterial species from diesel oil and 5 bacterial species from turbine oil (Table 13). All the pure colonies were frozen in duplicate and kept at -80 ° C for future growth and sequencing of DNA for identification.

Table 13- Abundance of isolated strains obtained from microbial consortia derived from the different sampling sites and degrading different hydrocarbons. First letter, D-Douro; M-Minho; C-Coast and second letter, ; O-Crude oil; D-Diesel oil (δ- colonies were only found in the 10^{-3} dilution and are countless; λ- colonies that spread a lot, the count it's not possible, however we were able to isolate); T-Turbine oil.

Douro estuary	CFU/ml	Minho estuary	CFU/ml	Coastal beach	CFU/ml
DO1	$7*10^6$	MO1	$9*10^6$	CO1	$21*10^5$
DO2	$6*10^6$	MO2	$6*10^6$	CO2	$41*10^5$
DO3	$24*10^6$	MO3	$3*10^6$	CO3	$27*10^5$
DO4	$5*10^6$	MO4	$13*10^6$	CO4	$34*10^5$
DO5	λ	MT1	$66*10^5$	CO5	$28*10^5$
DT1	$37*10^6$	MT2	$12*10^5$	CT1	$32*10^4$
DT2	$2*10^6$	MT3	$11*10^5$	CT2	$8*10^4$
DT3	$5*10^6$	MT4	$4*10^5$	CT3	$20*10^4$
DT4	$13*10^6$	MT5	$6*10^4$	CT4	$16*10^3$
DD1	$36*10^6$	MT6	$16*10^5$	CT5	$41*10^3$
DD2	$13*10^6$	MD1	$22*10^6$	CD1	$16*10^6$
DD3	$59*10^6$	MD2	$6*10^6$	CD2	$122*10^5$
		MD3	$16*10^6$	CD3	$14*10^5$
		MD4	δ	CD4	$20*10^5$
		MD5	$7*10^6$		

3.3.7 Bacterial richness and diversity

Bacterial richness and diversity indexes were calculated from ARISA profiles for the sediment initial and for each sediment treatment at the end of the experiment. For bacterial richness (Fig. 23A) no significant ($p>0.05$) differences were found between the three location (Douro and Minho estuarine, sandy coastal beach). Comparing each treatments with the respective control (T15) or with the initial sediment, no significant ($p>0.05$) differences were found for bacterial richness. For diversity index (Fig. 25B), no significant ($p>0.05$) differences were observed between sampling sites. However, significant ($p<0.05$) differences were found comparing different treatment with the respective control (T15) and with the initial sediment. After 15 days of exposure to the different types of oil, diversity index decreased for all locals (Fig. 23B).

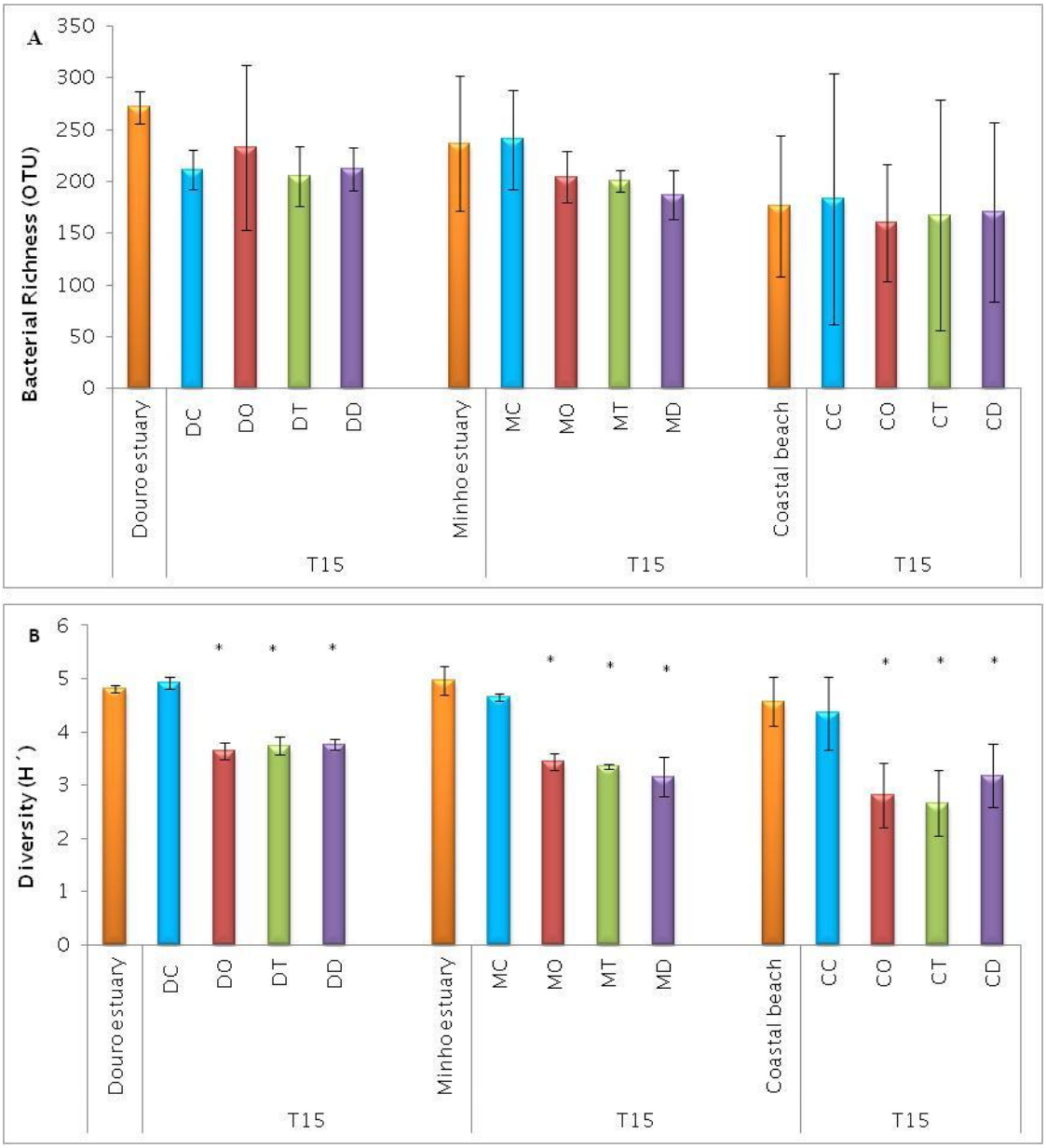


Fig. 23- Bacterial richness (A) and diversity (B) in sediment samples (n=3) based on ARISA profiles, in sediments from initial sediments (Douro estuary, Minho estuary and Coastal beach) and after 15 days of experiment (T15). First letter, D-Douro; M-Minho; C-Coast and second letter, C-Control; O-Crude oil; D-Diesel oil; T-Turbine oil. *significant differences were observed ($p < 0.05$) when comparing each treatment with the respective initial sediment and when comparing with the respective control.

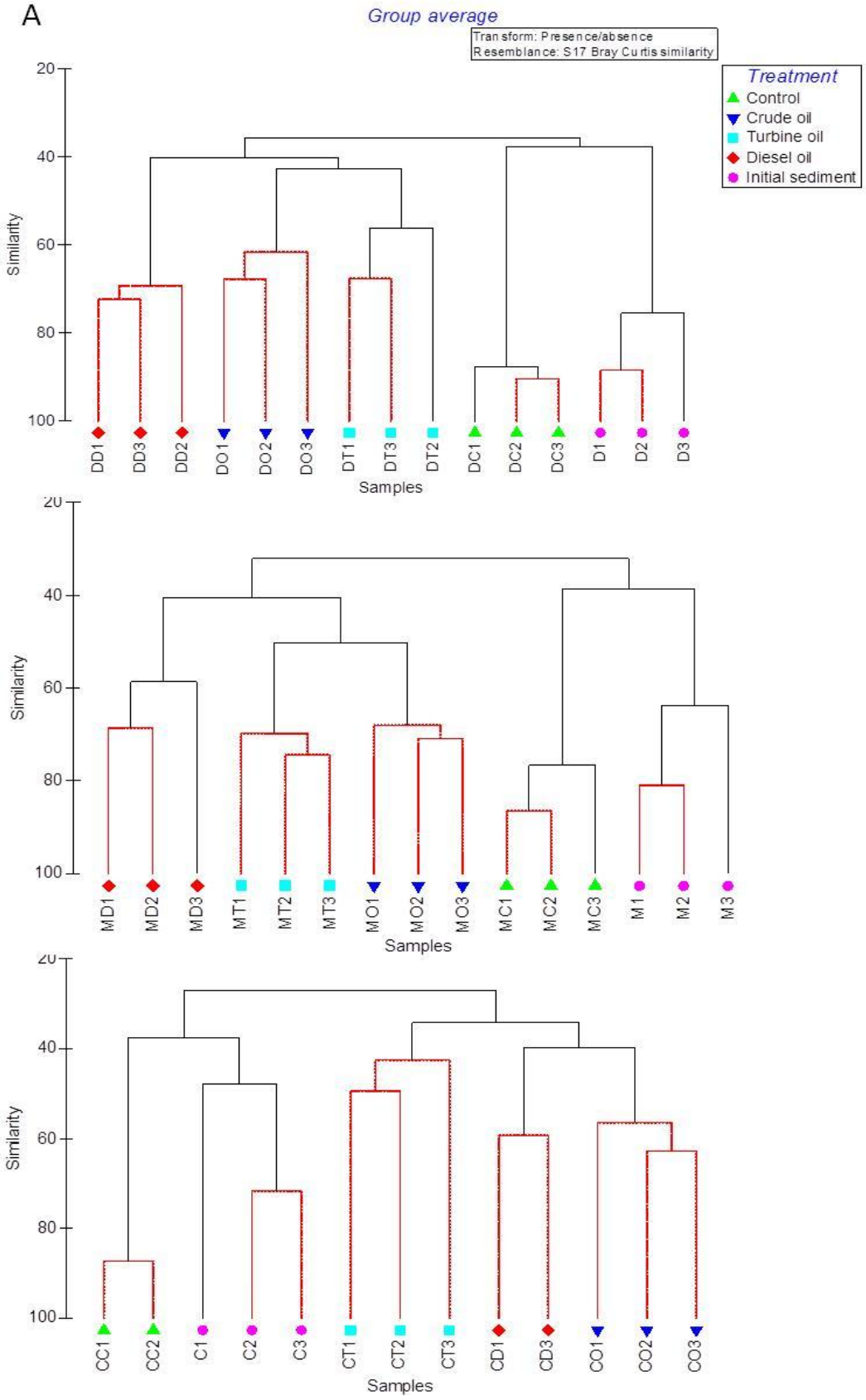
3.3.8 Bacterial community structure

ARISA analysis was performed to characterize the sediment bacterial community and to evaluate possible effects of different types of oil (crude, turbine and diesel oil) in that community. For each sample, ARISA fragment length (ALF) profiles were obtained. Different peaks correspond to different fragment lengths therefore different OTUs. The most relevant feature is given by the distribution of the different OTUs (bacteria phylotypes) among the different samples, since it corresponds to differences in their genetic structure as a function of the characteristics of the sediments and different type of oil contamination.

Various clustering of the samples (Fig. 24A) and MDS ordination (Fig. 24B) were made based on Bray Curtis similarities between samples, so as to evaluate changes in the microbial community structure. For each treatment, replicates were clustered together, being more similar (SIMPROF) between each sample, showing a good experimental replication. Thereby, it is possible to observe the different oil effect on community structure. To understand the factors responsible for the shaping of the bacterial community structure, analysis of similarities (two-way crossed ANOSIM) were performed (Table 14).

Through this test it was observed that not only the treatment but also the site have major influence on the community structure, presenting of values R close to 1. These R values were obtained for a significance level of 0.1%. Through the Pairwise Test, it was feasible to observe noteworthy differences between the various treatments and locations. Concerning the treatment factor, ANOSIM confirmed that the structure of the initial community is significantly affected by different oils. Regarding the location, ANOSIM confirmed significant differences between the different sampling sites. To sum up, results showed a significant effect of different types of treatment and significant differences between locations (Table 14). Therefore, all the factors tested in the experiment were relevant for the definition of the microbial community structure.

A


Group average
Transform: Presence/absence
Resemblance: S17 Bray Curtis similarity
Treatment
Control
Crude oil
Turbine oil
Diesel oil
Initial sediment
Similarity
20
40
60
80
100
DD1
DD3
DD2
DO1
DO2
DO3
DT1
DT3
DT2
DC1
DC2
DC3
D1
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D3
Samples
MD1
MD2
MD3
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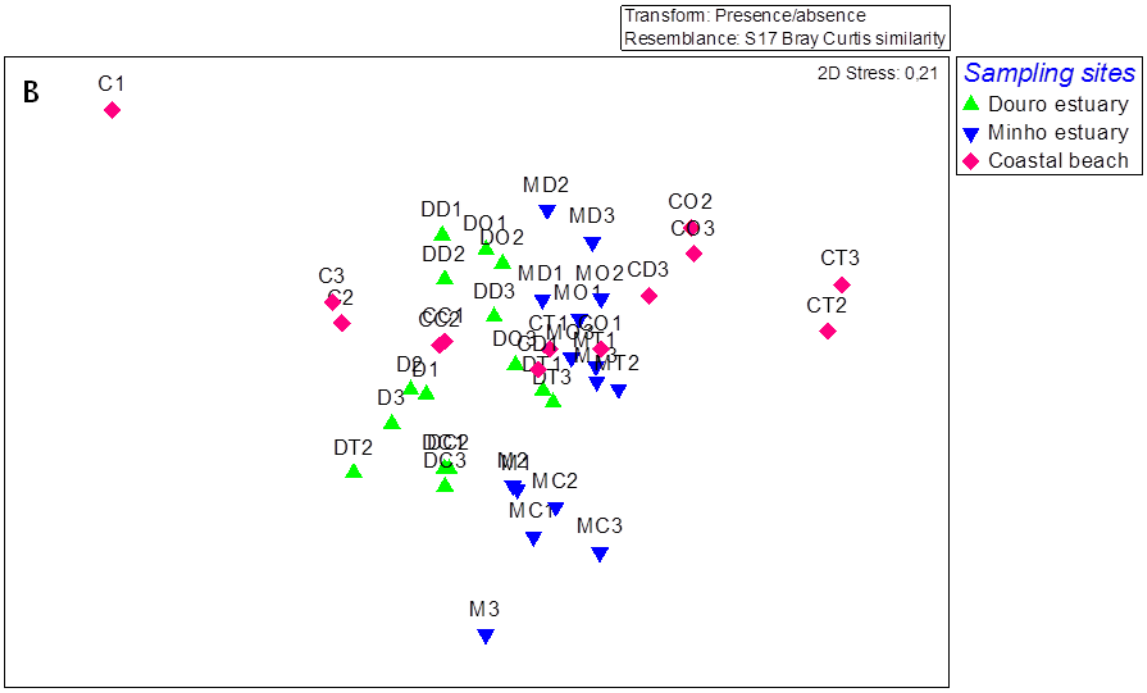

Fig. 24– Clusters analysis (A) and MDS ordination (B) based on Bray-Curtis similarities on the presence/absence matrix obtained from ARISA fingerprints of bacterial communities of the initial sediment and after 15 days of exposure to the different treatments.

Table 14– Global test of two-way crossed ANOSIM based on ARISA results from initial sediment and after 15 days of exposure of sediments to the different treatments.

	Statistic value (R)	**Significance level (%, p>0.05)**
Difference among local		
Global Test	0.978	0,1
Pairwise Tests		
Douro estuary, Minho estuary	1	0,1
Douro estuary, Coastal beach	0,983	0,1
Minho estuary, Coastal beach	0,983	0,1

Difference among treatments

Global Test	0.982	0,1
Pairwise Tests		
Control, Crude oil	1	0,2
Control, Turbine oil	0,959	0,2
Control, Diesel oil	1	0,3
Control, Inicial sediment	1	0,3
Crude oil, Turbine oil	0,963	0,1
Crude oil, Diesel oil	1	0,2
Crude oil, Inicial sediment	1	0,1
Turbine oil, Diesel oil	0,917	0,4
Turbine oil, Inicial sediment	1	0,2
Diesel oil, Inicial sediment	1	0,3

3.4 Discussion

The main purpose of the study was to assess the response of microbial communities to different types of oil contamination (crude oil, diesel oil and turbine oil). This response was evaluated in terms of their capacity to degrade hydrocarbons, microbial abundance, bacterial diversity indexes and bacterial community structure.

A relatively short time of experiments was selected (15 days). The overall microbial community response was probably not completed after 15 days. The heavy fraction of oil may persist for months or even years in the environment (Natter et al. 2012). Nevertheless, a clear response in terms of biodegradation of

some hydrocarbon fraction, namely the lighter ones, within this time frame was expected.

Regarding our results, in general, microorganisms from the different locations showed a potential for degradation (14% – 98%) of the different types of oil presenting significant differences between T0 and T15 TPH concentrations. Normally the crude oil adsorption is usually proportional to the OM, which was the case. In the case of the other two oils, this is not verified. Furthermore, the different sediments presented different absorption capacity for compounds different. Silva et al. (2015) observed that the selected microbial consortium present high ability to degrade diesel oil constituents and the maintenance of appropriate conditions leads to transformation of this oily source into less toxic compounds. The microbial consortium removed over 69% of the priority constituents from diesel oil in only one week. Our results are in agreement with this study, after a period of 15 days, autochthonous microorganisms had the ability degrade in the range 76% – 98% of the diesel oil.

For the turbine oil, autochthonous microorganisms had the ability to degrade in the range 14% – 86%. Hosokawa et al. (2010) also showed that the microbial consortium was able to degrade approximately 90% of turbine oil in five days. In a study that uses a soil from a petroleum refinery in a 7 month experiment, Couto et al. (2011) reported that indigenous microorganisms were able to consume, as a carbon source, most of the available turbine oil in a relatively short period of time.

Reis et al. (2014) evaluated the bioremediation potential of microorganisms from intertidal sediments of a sandy beach affected by a major oil spill. After a period of 15 days, autochthonous microorganisms had the ability to degrade up to 85% of the total petroleum hydrocarbons, increasing their abundance and changing the community structure. Our results are in agreement with this study, after a period of 15 days, autochthonous microorganisms had the ability to degrade in the range 30% – 89% of the crude oil.

Overall, microorganisms from the different locations showed a potential for degradation of the different types of oil. Our results are in agreement with the study by Horel et al. (2012), in which, it was noted a growth of microorganisms degrading hydrocarbons, which took the biodegradation of crude oil and diesel fuel. Furthermore, they reported that the addition of nutrients increases the rate

of degradation. Thus, autochthonous microorganisms had the ability to degrade the crude oil and its derivative, increasing their abundance and changing the community structure.

Significant differences ($P<0.05$) among hydrocarbon degrading microorganisms were observed between the sampling site (initial sediment) and between sediment from each treatment. After 15 days of exposure to the different types of oil, all microbial communities increased their hydrocarbon degrading microorganisms. Our results are in agreement with Ribeiro et al. (2015b) who also found an increase in HD abundance in sediments contaminated with petroleum. Couto et al. (2012) searched an appropriate biological approach for recovering a refinery soil contaminated with petroleum hydrocarbons reporting an increase in MPN of crude oil degraders and turbine oil degraders. Also, it was observed in our results an increase in MPN of diesel oil degraders as mentioned above.

In relation to TCC, in general, the Minho estuary and Coastal beach were observed significant ($P < 0.05$) difference in the various samples, both initial sediment like in all treatments, except for the Douro estuary, where it is only possible to consider large differences between the initial sediment and sediment contaminated with oil.

For all sampling sites, we isolated bacterial species of different treatments. In a recent study, Silva et al. (2015) tested around the 86 microorganisms. They observed the bacteria *Staphylococcus saprophyticus UFPEDA 800* and *Serratia marcescens UFPEDA 839* and the yeasts *Rhodotorula aurantiaca UFPEDA 845* and *Candida ernobii UFPEDA 862* displayed rapid diesel oil degradation, and when used together as a consortium, there was no antagonistic activity. The consortium of these 4 species removed the priority constituents from diesel oil in only one week when temperature, C:N ratio, inocula quantity, pH, shaking, and aeration were optimized. Hosokawa et al. (2010) also was able to isolate from a consortium from turbine oil, fourteen bacterial species.

Since one of the main purposes of the study was to assess the response of microbial communities to bioremediation treatments, bacterial community structure was assessed. The technique used, ARISA, provided a high-resolution fingerprinting methodology to monitor changes in microbial community structure

and estimation of microbial richness and diversity. Very recent studies consider that bacterial ARISA is still a powerful tool for analyzing bacterial communities, especially for controlled experiments (Tkacz et al. 2015; Purahong et al. 2015). Gobet et al. (2013) reported that ARISA has significantly contributed to advancing microbial community ecology and it is very well suited for a general overview of changes in the abundance of bacterial types. The most relevant information is given by the distribution of the different OTUs (bacteria phylotypes) in the different samples. The multivariate analysis of all generated ARISA profiles allowed detection of several differences in terms of community structure between treatments and between sampling sites which were confirmed by analysis of similarities (two-way crossed ANOSIM). Our study was consistent with coastal sediments studies that also showed that the structure of the bacterial community may change substantially when exposure to crude oil (Païssé et al. 2010). Ribeiro et al. (2015b) indicated that sediment characteristics were the most important factor shaping the bacterial community structure, followed by the other factors such as petroleum contamination and bioremediation treatments. Despite no significant differences where observed in terms of bacterial richness, bacterial diversity significantly decreased in all locations at the end of the experiment. This decrease in diversity may be due to a deleterious effect of hydrocarbon pollution in the most sensitive species and dominance of species with metabolic capacity to degrade hydrocarbons. Also Ribeiro et al. (2015b) reported that petroleum contamination led to the stimulation of HD within the total bacterial assemblage, contributing to a diversity reduction.

3.5 Conclusions

To sum up, the results obtained indicate that the studied autochthonous microbial communities were able to adapt to the different types of oil by increasing their abundance and changing their community structure. Multivariate analysis showed significant effect of type of treatment on the microbial community structure, and significant differences between sampling sites. For all sampling sites, bacterial species were isolated from the different treatments.

Thus, knowledge of microbial diversity and metabolism in oil-polluted sites can be helpful for bioremediation of oil spills, as human intervention can be planned for cleaning up oil pollution by using specific microbial consortia.

Chapter 4

GENERAL DISCUSSION AND CONCLUSIONS

4. General discussion and conclusions

4.1 General discussion

Estuarine ecosystems are among the most productive but also the most sensitive to contamination (Mucha et al. 2011) being their preservation extremely important. They have unique physical conditions that support extremely diverse organisms and offer essential relations to near ecosystems (Sun et al. 2012).

Several methodologies have been proposed for ecosystems recovery from contamination events, including bioremediation. For proper implementation of this technique it is necessary to have prior knowledge about the capacity of the autochthonous microbial communities to biodegrade petroleum hydrocarbons.

The aim of this study was to understand the potential of microbial communities for hydrocarbon degradation along the NW Portuguese Coast. For that, two different works were performed.

The first study aimed to characterize the hydrocarbon degradation potential of autochthonous microorganisms collected along the northern Portuguese Coast with different degrees of contamination. The sediment analysis allows the determination of the quality of aquatic ecosystem since they are a reservoir of pollutants (Wu et al. 2014).

It was observed that the Douro estuary presented a greater number of degrading microorganisms, which may be related to a previous adaptation of these communities to pollution. In addition, the Minho estuary and sandy coastal beaches also presented microorganisms with ability to degrade petroleum hydrocarbons. Thus, in a contaminated environment with oil, the proportion of hydrocarbons and degrading microorganisms increases rapidly, so the amount of these microorganisms, which have the ability to use the oil as a source of carbon and energy, normally, reflects the degree of contamination of the ecosystem (Almeida et al. 2013b).

It is clearly observed some relationships between HD, TCC, TPHs and some characteristics of sediments, particularly OM content. For example, sediments with higher OM content, in estuarine areas, have also higher TCC. On the other hand, these same sediments present a lower abundance of HD, despite high

concentrations of TPHs. This last observation may be associated with the fact that the OM content may affect the availability of hydrocarbons preventing its degradation (Almeida et al. 2013a). They demonstrated this relationship, in which the high OM content of sediments may play an important role in adsorption, control, distribution and hydrocarbon concentration. Within Douro estuary it was observed a greater abundance of HD at the sandy sample. This result suggests that the coarser particles can provide more favorable conditions for the growth of HD.

The bacterial community structure was also assessed. Our results showed no significant differences in terms of bacterial richness and diversity indexes between the three zones (Coast, Douro and Minho). Nevertheless, it was observed a differentiation of the microbial community structure with location confirmed by ANOSIM. The sites were grouped by their characteristics. These data suggest that sediment characteristics probably contributed for the dissimilarity between microbial community structures. In order to analyze the relationships between environmental and biological variables, the BIO-ENV analysis was performed. BIO-ENV procedure enables the selection of the abiotic variable subset that maximizes the rank correlation between biotic and abiotic (dis)similarity matrices. This analysis selected both sediment structure (e.g. organic matter content) and contamination (e.g. Tetrachloroethylene) as environmental parameter with greater correlation to the microbial community.

Therefore, at the selected sites, the characterized autochthonous microbial communities have the potential to degrade hydrocarbons, being important to assess experimentally their ability to bioremediation of these pollutants. Thus, a second study was carried out to assess the response of microbial communities to different oil contamination (crude oil, diesel oil and turbine oil). The sampling sites were chosen according to the sediment and microbial community characterization on a previous study (Chapter2). Based on these criteria, one sampling location was selected in each area (Douro and Minho estuary and Coastal beach) to be considered for next study (Chapter 3).

The response of microbial community for bioremediation of sediments contaminated with different types of oil was assessed in terms of community structure, abundance, and capacity to degrade hydrocarbons. After a period of 15 days, different sediments contaminated with different oils types, positively

influence the microbial community by increasing total microbial abundance and promote the development of HD populations. In general, microorganisms from the different locations showed a potential for degradation (14% – 98%) of the different types of oil presenting a significant differences between T0 and T15 TPH concentrations. For all sampling sites, we isolated bacterial strains from different treatments to be identified in the future.

Regarding bacterial richness, no significant differences between the beginning and the end of the experiment were observed in all sites. In terms of bacterial diversity it was observed a significant decrease in all locations at the end of the experiment. This decrease in diversity may be due to a deleterious effect of hydrocarbon pollution in the most sensitive species and a dominance of species with metabolic capacity to degrade hydrocarbons. Bacterial community structure showed differences between locals and treatments, which were confirmed by ANOSIM. Thus, all the treatments tested in the experiment had a significant impact on microbial community structure. Therefore, suggesting that the main effect of oils was the stimulation of HD, increasing the relative importance of these bacteria within the assemblage, which led to the obtained diversity reduction (Ribeiro et al. 2015b). Thus, results showed an adaptation process of microbial community.

4.2 Conclusions

Results from our study show that higher abundance of hydrocarbon degrading microorganisms was detected in the Douro estuary, which was also the location with higher levels of total petroleum hydrocarbons. Nevertheless, hydrocarbon degrading microorganisms were found in all collected sediments, despite the different degrees of petroleum hydrocarbon contamination. Thus, at the selected sites, the characterized autochthonous microbial communities have the potential to degrade hydrocarbons. Relatively the second work, results showed that all microbial communities increased their microbial abundance. The autochthonous microorganisms from the different locations presented capacity to adapt to the different types of oil and potential for bioremediation of contaminated sediments.

Thus, knowledge of microbial diversity and metabolism in oil-polluted sites can be helpful for bioremediation of oil spills, as human intervention can be planned for cleaning up oil pollution by using specific microbial consortia.

Chapter 5

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